85. (once amended) The polypeptide of claim 84, wherein the non-native disulfide bond is formed with a cysteine residue at one or more of positions 23, 81, 22, 82, 53 or 70.

86. (once amended) The polypeptide of claim 85, wherein the non-native disulfide bond is formed between cysteine residues substituted at one or more of the following pairs of positions: 22 and 82; or 23 and 81.

87. (once amended) A polypeptide comprising any one of Seq. ID Nos. 24, 26, 28, 30, 32 or 35-53, wherein the polypeptide is modified to have a non-native disulfide bond and more than seven non-native essential amino acid residues.

96. (new) The polypeptide of claim 10 further comprising the pair of substitutions that is V53C and V70C.

97. (new) The polypeptide of claim 22, further comprising the pair of substitutions that is V53C and V70C.

REMARKS

Claims 9-14, 16-22, 24, 28-32, 54-56, 59-62, 66, 69, 71, 72, 74, 76 and 78-87 have been amended. New claims 96 and 97 have been added. Support for the amendments and new claims can be found in the specification, particularly the pages as noted herein.

Attached hereto as **Appendix C** is a marked version of the claims as amended by the current amendment. The attached pages are captioned "Marked Version of Complete Claims."

Attached hereto as **Appendix D** is a marked version of the portions of the specification as amended by the current amendment.

The basis for these amendments is set forth below.

DRAWINGS

Corrected Figures 1 and 2 with the required margins are submitted with this Amendment and Response. Figures 1 and 2 have also been amended as stated below. If any additional correction to Figures 1 and 2 is required Applicants request specific direction.

COMPLIANCE WITH THE SEQUENCE RULES

Applicants have submitted amended Figures 1 and 2 which now indicate the Seq. ID numbers of each of the sequences listed therein. The polypeptide sequence of BHLX in Figure 1 has been added as Seq. ID no. 33, and the polypeptide sequence of BHLXP has been added as Seq. ID no. 34. The polypeptide sequence of homologs 1-19 in Figure 2 have been added as Seq. ID Nos. 35-53, respectively. These sequences are also publicly available, as indicated by the genbank accession numbers for homologs 1-19 as shown on P. 5, lines 18-38 of the specification.

Applicants point out that certain genbank sequences included in the original application have become non-functional on genbank for reasons not known to the Applicants. However, Applicants have identified functional alternative sequences on genbank as follows: For Seq. ID no. 35, A01293 has been replaced with gi:68800; For Seq. ID no. 37, S37493 has been replaced with 475922; For Seq. ID no. 42, S33547 has been replaced with gi:19913; For Seq. ID no. 47, A01291 has been replaced with P01052. In addition, Applicants point out that sequence A39547 is still functional on genbank, and although the sequence appears to have been intentionally withdrawn from genbank, the sequence was on genbank at the time the application was filed. It also may be possible to use this accession number to identify the sequence in archived genbank records or in the patent application that referenced such sequence.

In any event, such sequences were shown in Figure 2 of the specification as filed and no new matter has been added.

Applicants have also amended the sequence listing to include the Seq. ID Nos. as shown below:

Oligonucleotide Sequence	Seq. ID No.
N4394	54
N4395	55
N4396	56
N4397	57
N5045	58
N5046	59
N13561	60
N13562	61
N13563	62
N13564	63
N13565	64
N13905	65
N14471	66
N14472	67
N13771	68
N22098	69
N22099	70
N23923	71
N23924	72
N26671	73
N26672	74

Each of these oligonucleotide sequences were previously shown on pages 50-54 of the specification. Applicants hereby state that: (1) the amendments, made in accordance with 37 CFR §1.825(a), included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages 5, 50-54 and Figures 1 and 2, (2) the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(b), is the same as the amended Sequence Listing, and (3) no new matter has been added.

Objections to the Specification

7. The Examiner has objected to the inappropriate citation of an internet address on P. 12, line 17. Applicants have amended such paragraph so that such citation is in accordance with MPEP 707.05(e). Applicants submit that this objection has been overcome.

8. The Examiner has objected to the Abstract for not completely describing the disclosed subject matter. The Examiner suggested the inclusion of the full name of the protein and its source(s) for completeness. Applicants thank the Examiner for this suggestion and have amended the Abstract accordingly. Applicants submit that this objection has now been overcome.

9. The Examiner has objected to the amendments to the claims under 35 U.S.C. 132 on the basis that they introduce new matter into the disclosure. The Examiner has listed 18 points related to new matter, lettered (a)-(r).

9. a-d

With respect to new matter objections a) through d), Applicants have amended claims 9, 54 and 55 to refer to the previous ranges of 5-15 mole % methionine, 6-25 mole % threonine and 4-9 mole % tryptophan. While Applicants emphasize that the application broadly teaches various embodiments of modified CI-2 that extend well beyond the specific examples taught and specific ranges recited in the specification, Applicants have amended these claims in order to expedite prosecution. Accordingly, these objections have been rendered moot.

9.e

The Examiner objects to the language in claim 59, "at least 60% sequence identity..... Seq. ID No.: 2" and "about 15% or more lysine". Applicants point out that polypeptide variants are encompassed within the inventions, see P. 26, line 19, and polypeptide variants are defined on page 12, lines 1-3 as at least about 55%, 60%, 70%, 80%, or preferably at least about 90% and more preferably at

least about 95% sequence identity to the modified protein. In addition, based on a 51 amino acid subsequence, 15 mole % or more lysine is more than 7 lysine residues. A number of embodiments are contemplated with more than 7 lysine residues. For example, Seq. ID No. 12 has 21 lysine residues. Seq. ID Nos. 33 and 34 represent numerous embodiments with more than 7 lysine residues. The specification, on p. 44, line 19, teaches embodiments of the invention comprising substituted CI-2 like polypeptides, or truncated versions thereof substituted to contain more than 7 non-native essential amino acid substitutions. To clarify the language of claim 59 in order to expedite prosecution, "about 15 mole % or more lysine" has been replaced with "more than seven lysine amino acid residues." Applicants submit that this objection has now been overcome.

9. f

The Examiner objects to the language in claim 60, "about 20 mole % or more lysine". Based on a 51 amino acid subsequence, about 20 mole % or more lysine is more than 10 lysine residues. A number of embodiments are contemplated with more than 10 lysine residues. For example, Seq. ID No. 12 has 21 lysine residues. Seq. ID Nos. 33 and 34 represent numerous embodiments with more than 10 lysine residues. The specification, on p. 44, line 20, teaches embodiments of the invention comprising substituted CI-2 like protein with more than 10 non-native essential amino acid substitutions. To clarify claim 60 in order to expedite prosecution, "about 15 mole % or more lysine" has been replaced with "more than ten lysine amino acid residues." Applicants submit that this objection has now been overcome.

9. g

The Examiner objects to the language in claim 61, "at 4 to about 34 positions" and "Seq. ID No. 2 positions 19-53 and 63-83". Applicants respectfully traverse this objection.

Applicants emphasize that they have recognized that Seq. ID No. 2 is a useful protein for modification to increase essential amino acid content. Applicants have taught numerous embodiments of the invention, including the

formulas of Seq. ID Nos. 33 and 34, shown in Fig. 1. On page 44, line 18, Applicants teach that substituted CI-2 may have more than 4 substitutions. Applicants teach embodiments of the invention that have up to 35 substitutions with non-native essential amino acids. For example, see Seq. ID. No. 34 and the specification, page 41, line 30, and page 44, line 26. Notwithstanding, Applicants have amended claim 61 to require modification in "more than four positions," thereby not specifying the range "4 to about 34." Support for more than four is found throughout the specification and specifically at page 44, line 18. Applicants have further amended the claim to require that the amended polypeptide have at least 30% sequence identity to any one of Seq. ID Nos. 4 or 20. Support for a polypeptide with at least 30% sequence identity to Seq. ID Nos. 4 or 20 is found in the specification, page 8, lines 16-22, where the specification teaches that a "CI-2 like" polypeptide refers to a polypeptide ...of at least 30% amino acid sequence identity with corresponding region of Seq. ID Nos. 2 or 4 or 20." The Applicants clearly envisioned the embodiments of the invention encompassed by claim 61, embodiments with at least 30% sequence identity with Seq. ID Nos. 4 or 20 as now claimed.

With respect to the positions of the amino acid residues being modified, Applicants teach, on page 46, lines 24-26, that modification in the active site loop area by amino acid substitution or other means destroys the hydrogen bonding and changes or reduces the protease inhibitor activity of BHL. Applicants teach, for example, on page 40, lines 10-12 of the specification, that the active site loop region encompasses an extended loop region from about amino acid residue 53 to about amino acid residue 70. Applicants have disclosed embodiments that have elevated essential amino acid content but do not have reduced protease inhibitor activity (page 41 lines 3-5 of the specification). From Applicants teaching, one of ordinary skill in the art would understand that such embodiments would comprise modified CI-2 with substitutions made outside of the active site loop. On page 46, line 24, to page 47, line 2, Applicants teach that "modification in the active site loop area by amino acid substitution or other means, destroys the hydrogen bonding and changes or reduces the protease inhibitor activity of BHL." Embodiments without reduced protease inhibitor activity would include

those without modifications in the active site loop area, and such embodiments are the subject of claim 61.

Applicants also teach, on page 40, line 20, that the first 18 residues in the wild type CI-2 (Seq. ID No. 2) do not assume any ordered conformation and may be truncated. Similarly, Applicants teach on page 43, line 31, to page 44, line 1, that in one embodiment "the truncated version excludes the region corresponding to the amino terminal 17 or 18 amino acids of Seq. ID No. 2." Claim 61, as amended, is directed at defining the inventions by reference to the positions on CI-2 (19-53 and 70-83) other than the first 18 unordered residues or the active site loop region at amino acid residues 54-69. Modifications in these regions were contemplated by Applicants at the time the application was filed and the Application supports not making modifications at position 1-18 and 54-69. Applicants submit that claim 61, as amended, does not contain new matter and the objection should be withdrawn.

9.h

The Examiner objects to the language in claim 72, "at least 4 to about 34 positions" and "sequence ID No. 2 positions 19-83." Applicants have recognized that Seq. ID No. 2 is a useful protein for modification to increase essential amino acid content. Applicants have taught numerous embodiments of the invention, including the formulas of Seq. ID Nos. 33 and 34, shown in Fig. 2. On page 44, line 18, Applicants teach that substituted CI-2 may have more than 4 substitutions. As noted in the response to objection to claim 61 in 9.g. above, Applicants teach embodiments of the invention that have up to 35 substitutions with non-native essential amino acids. Notwithstanding, in order to expedite prosecution Applicants have amended claim 72 in the same manner specified for claim 61. Support for these amendments is as set forth in 9. g above.

Further, Seq. ID No. 2 positions 19-83 is supported by the specification because it claims the sequence exclusive of the first 18 unordered residues. The characterization of the sequence exclusive of the first 18 unordered residues is further supported by the Seq. ID's Nos. 4, 6, 8, 10, 14, 16, 18, 20, 33 and 34 which exclude these unordered residues (see Fig. 2) and for the reasons

specified in 9.g above. Also, as used in the claim, Seq. ID No. 2 positions 19-83 are used to describe amino acid positions in a clear manner. The claim is not directed to Seq. ID No. 2, but to sequences that are modifications of Seq. ID Nos. 2, 4, 6, 8, 10, 12, 14, 18 and 20, each of which are described in the specification as filed. Applicants submit that claim 72, as amended, does not contain new matter and the objection should be withdrawn.

9.i

The Examiner objects to the language in claim 74, "at 6 to about 34 positions" and "sequence ID No. 2 positions 19-53 and 63-83."

Applicants have amended "at 6 to about 34 positions to "more than 7 positions", and have amended "63-83" to "70-83". Further, as noted in 9.e above, the specification teaches embodiments of the invention comprising more than 7 non-native essential amino acids. Applicants have added the same limitation to 30% sequence identity as added to claims 61 and 72, and this limitation is supported by the specification for the reasons stated in 9.g above. Applicants submit that claim 74, as amended, does not contain new matter and the objection should be withdrawn.

9.j

The Examiner objects to the language in claim 76, "at least 23 amino acids in length" and "at least 11 non-native essential amino acids" and "sequence ID No. 2 positions 19-83".

Claim 76 has been amended to delete "at least 23 amino acids in length." Applicants point out that polypeptides with more than 10 non-native essential amino acid residues are taught in the specification on page 41, line 24, page 44, line 4, and page 44, line 20. Seq. ID No. 2 positions 19-83 is supported by the specification because it claims the sequence exclusive of the first 18 unordered residues as discussed in 9.g and 9.h above. Applicants have added the same limitation to 30% sequence identity as added to claims 61 and 72 and supported by the specification for the reasons stated in 9 g above. Applicants submit that claim 76, as amended, does not contain any new matter.

9.k

The Examiner objects to the language in claim 78, "at least 23 amino acids in length" and "at least 64% identity to... positions 19-83 in sequence ID No. 2" and "at least one cysteine residue in... positions 19-83 in sequence ID No. 2".

Applicants have amended claim 78 so that it now reads upon a polypeptide having at least 60% identity to Seq. ID No 4. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants. For example, pages 17-18 of the specification teach numerous ways to modify the nucleotide sequence in order to obtain polypeptide sequence variants. Specifically, page 17, lines 18-21 teach that "Variants included in the invention may contain individual substitutions, deletions or additions to the nucleic acid or polypeptide sequence." With respect to the addition of disulfide bonds, Example 3 on page 56, line 32, to page 57, line 15, teaches the addition of disulfide bonds to the polypeptide. Modification of a CI-2 like protein to contain a disulfide bond is also described on page 43, lines 7-14 of the specification, in which Applicants state, "The substituted CI-2 like protein is made more stable by the addition of disulfide bonds. In one embodiment from one to less than 5 disulfide bonds are added." Further, the term "at least 23 amino acids in length" has been deleted. Applicants recognize that short sequences with high percentage identities to segments of Seq. ID No. 4 may exist. However, such short sequence would not fall within the scope of the claim unless they had at least 60% identity to the full length sequence of Seq. ID No. 4. Applicants submit that no new matter has been added to claim 78 as amended and the objection should be withdrawn.

9.l

The Examiner objects to the language in claim 79, "at least 23 amino acids in length" and "at least 60% identity to... positions 19-83 in sequence ID No. 2" and "at least one cysteine residue in... positions 19-83 in sequence ID No. 2".

Applicants have amended claim 79 so that it now reads upon a polypeptide having at least 60% identity to Seq. ID No 6. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as described in 9.k above. As described in 9.k above, Example 3 on page 56, line 32, to page 57, line 15, teaches the addition of disulfide bonds to the polypeptide. Further, the term "at least 23 amino acids in length" has been deleted. Applicants recognize that short sequences with high percentage identities to segments of Seq. ID No. 6 may exist. However, such short sequence would not fall within the scope of the claim unless they had at least 60% identity to the full length sequence of Seq. ID No. 6. Applicants submit that no new matter has been added to claim 79 as amended and the objection should be withdrawn.

9. m

The Examiner objects to the language in claim 80, "at least 74% identity." Applicants have amended claim 80 so that it now reads upon a polypeptide having at least 70% identity to Seq. ID No 6. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as noted above. Applicants submit that no new matter has been added to claim 80 as amended and the objection should be withdrawn.

9.n

The Examiner objects to the language in claim 81, "at least 23 amino acids in length" and "at least 57% identity to... sequence ID No. 8" and "at least one cysteine residue in... positions 19-83 in sequence ID No. 2".

Applicants have amended claim 81 so that it now reads upon a polypeptide having at least 60% identity to Seq. ID No 8. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as noted above. Further, Example 3 on page 56, line 32, to page 57, line 15, teaches the addition of disulfide bonds to the polypeptide and the term "at least 23 amino acids in length" has been deleted. Applicants recognize that short sequences with high percentage identities to segments of Seq. ID No. 8 may exist. However, such short sequence would not fall within the scope of the claim unless they had at least 60% identity to the full length sequence of Seq. ID No. 8. Applicants submit that no new matter has been added to claim 81 as amended and the objection should be withdrawn.

9.o

The Examiner objects to the language in claim 82, "at least 67% identity." Applicants have amended claim 82 so that it now reads upon a polypeptide having at least 70% identity to Seq. ID No 8. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as noted above. Applicants submit that no new matter has been added to claim 82 as amended and the objection should be withdrawn.

9.p

The Examiner objects to the language in claim 83, "at least 23 amino acids in length" and "at least 57% identity to... sequence ID No. 10" and "at least one cysteine residue in... positions 19-83 in sequence ID No. 2".

Applicants have amended claim 83 so that it now reads upon a polypeptide having at least 60% identity to Seq. ID No 10. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as noted above. Further, Example 3 on page 56, line 32, to page 57, line 15, teaches the addition of disulfide bonds to the polypeptide and the term "at least 23 amino acids in length" has been deleted. Applicants recognize that short sequences with high percentage identities to segments of Seq. ID No. 10 may exist. However, such short sequence would not fall within the scope of the claim unless they had at least 60% identity to the full length sequence of Seq. ID No. 10. Applicants submit that no new matter has been added to claim 83 as amended and the objection should be withdrawn.

9.q

The Examiner objects to the language in claim 84, "at least 67% identity." Applicants have amended claim 84 so that it now reads upon a polypeptide having at least 70% identity to Seq. ID No 10. The specification, on page 12 lines 1-3, teaches that "polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80% ... sequence identity to the modified protein." The specification teaches methods of obtaining sequence variants as noted above. Applicants submit that no new matter has been added to claim 84 as amended and the objection should be withdrawn.

9.r

The Examiner objects to the language in claim 87, "at least 20 amino acids in length" and "modified to have at least seven non-native essential amino acid residues."

Applicants have amended claim 87 to refer only to specific Seq. ID Nos. disclosed in the specification. Modification of CI-2 like polypeptides with more than 7 non-native essential amino acid residues is disclosed in the specification. For example on page 44, line 19, the specification states that in one

embodiment "the substituted CI-2 like protein has more than 7" substitutions. Modification of a CI-2 like protein to contain a disulfide bond is taught on page 43, lines 7-14, of the specification "The substituted CI-2 like protein is made more stable by the addition of disulfide bonds. In one embodiment from one to less than 5 disulfide bonds are added." The addition of disulfide bonds is also taught in Example 3 as noted above. Applicants submit that no new matter has been added to claim 87 as amended and the objection should be withdrawn.

Objections to the Claims

10) The Examiner has objected to claims 54 and 55 under 37 CFR 1.75(c) as being of improper form. Applicants have amended the parent claim (Claim 9) in accordance with the Examiners 112, second paragraph, rejection concerning the confusion about the mole percent combinations and have amended claims 54 and 55 (and parent Claim 9) to place them in proper dependent form (see also paragraph number 12 below). Applicants submit that this rejection has now been overcome.

Claim Rejections – 35 USC Section 112

11) Claims 9-20 and 54-89 were rejected under 35 U.S.C. 112, second paragraph, for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Examiner states that "The persistent use of numerals in the claims, when referring to (a) sequence length, (b) mole percent, (c) positions in defined sequences, (d) number of modifications, and (e) particular modifications, renders the claims very difficult to interpret. The Examiner suggests using numerals for mole percent (15-35 mole % lysine), positions in defined sequences (positions 1,8, etc.) and particular modifications (T22C); however, the Examiner recommends amending the claims to use words for sequence length (fifty amino acids in length) and numbers of modifications

(modified to contain seven or more). Applicants thank the Examiner for these suggestions and have amended the claims accordingly.

12) Claims 9 and 54-55 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "The claim language of Claim 9 is confusing as to whether the "composition.. .or in combinations thereof" requires that all the listed mole percents be present in a polypeptide or if only one mole percent is required." The Examiner has suggested that if only one mole percent is required, Claim 9 be amended to include the following phrase ---altered to have a composition selected from the following--- and the Markush group should be joined by ---and-- (not "or" as claimed presently). The Applicants have amended Claim 9 accordingly and thank the Examiner for the suggestion. Applicants have also amended the word "altered" to the word "modified" to be consistent with terms used in other claims. Claims 54-55 are of more limited scope than Claim 9, because, for example, a polypeptide meeting the requirements of Claim 9 by virtue of containing 15-35 mole % lysine would not meet the requirements of Claim 54 if it did not have 5 –15 mole % methionine. Applicants submit that this rejection has now been overcome.

13) Claims 9, 54, 55, 59, and 60 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner stated that "The concept of "mole %" is unclear as to whether whole numbers are required." The Examiner suggested inserting ---at least 12 mole percent--- to clarify in the instant claims if whole integers are required. The Applicants thank the Examiner for this suggestion and have amended the claims that refer to mole % accordingly.

14) Claims 10-18 and 56 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly

claim the subject matter which applicant regards as the invention. The Examiner states that "Claim 10 claims not only corresponds to Seq. ID No. 2, but also truncated versions of Seq. ID No. 2. However, the positions for modification extend almost the entire length of Seq. ID No. 2. Thus, the extent to which truncated forms fall within the scope of the instant claims is unclear." Applicants respectfully traverse this rejection. The positions for modification do not require a modification to be made at every one of those positions, only that seven or more non-native essential amino acids be substituted in at least 7 of those positions. As is taught in the specifications, Seq. ID. No. 2 is the non-truncated form of wild type CI-2, while Seq. ID. No. 4 is the truncated form of wild type CI-2. While Seq. ID. No. 2 contains from 1-83 amino acids, Seq. ID. No. 4 represents wild type CI-2 truncated to remove the signal peptide represented by the first 18 amino acids. Seq. ID. No. 4 thus contains the same amino acids as those amino acids at positions 18 to 83 of Seq. ID. No. 2. (See Figure 1 of the specification) Claim 10 has been amended to clarify that the truncated version of Seq. ID. No. 2 referred to in Claim 10 is represented by Seq. ID. No. 4. Further, while Seq. ID. No. 4 does not contain amino acids at positions corresponding to the positions 1, 8, 11, 17 and 18 in Seq. ID. No. 2, it does contain amino acids at positions corresponding to positions 19, 20, 22, 23, 31, 34, 38, 40, 41, 47, 49, 56, 58, 59, 60, 61, 62, 63, 65, 67, 69, 73, 75, 76, 78, 79, 81 and 82 in Seq. ID. No. 2. Any seven or more of the amino acids at those positions could be modified in accordance with Claim 10. In light of these arguments and amendments, Applicants respectfully request withdrawal of this rejection.

15) Claims 10-18, 56, and 61-71 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "The phrase "modified to contain" is confusing since the sequences claimed contain essential amino acids prior to modification. It is unclear whether an essential amino acid needs to be added by modification to the sequence or if, once modified, the sequences must *still* contain essential amino acids." Applicants respectfully traverse this rejection. The invention and

teaching in the specification are directed to altering the composition of CI-2 in order to increase the nutritional value. In the context of Claims 10, 61, 72, 74, 76, 78, 79 and 81, as amended, the phrase "modified to contain at least (X number) of non-native essential amino acids" means that the sequence has been modified to replace native amino acids with non-native essential amino acids. After modification, the sequence will still contain both native and non-native essential amino acids. However, at least the specified number of non-native essential amino acids must have been added to the sequence by modification. Similarly, in some of these claims the phrase "modified to contain at least one non-native disulfide bond" is used. This phrase means that a disulfide bond that did not exist in the polypeptide prior to modification must be added to the polypeptide as a result of modification. In light of these arguments, Applicants respectfully request withdrawal of this rejection.

16) Claims 10-25, 56, and 61-86 were rejected under 35 U.S.C. 112, second paragraph. as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "In Claims 10, 19, 21, 56, 61, 69, 72, 74, 76, 78, 79, 81, 83, 85, and 86, the phrase 'corresponding to' or 'correspond to' is confusing because the relatedness of Seq. ID No.2 to all other possible polypeptides is not clear. Particularly, how to identify which residues 'correspond' is unclear." Applicants respectfully traverse this rejection. Figure 1 of the specification provides a clear example of how to align sequences and compare amino acid positions corresponding to the positions of amino acids in Seq. ID No. 2. Methods of using computers to perform these types of sequence alignments are also well known in the art. For example, the programs GAP and PileUp, each available from available from the University of Wisconsin Genetics Computer Group, may be used to assist in this determination. Applicants point out that the Examiner has used computer methods of alignment to find the references cited in the claim rejections under 35 USC Section 102, and to align sequences in these references with corresponding positions in Seq. ID No. 2. Further, since polypeptides with at least 30% sequence identity to specific Seq. ID Nos. are

claimed, difficulty in comparing sequences with very low % sequence identity will be avoided. In light of these arguments, Applicants respectfully request withdrawal of this rejection.

17) Claim 11 was rejected under 35 U.S.C. 112. second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "Claim 11 limits the essential amino acid particular amino acids and their conservative substitutions. The instant specification defines conservative substitutions on page 10, lines 20-30. and defines conservative essential substitutions on page 10, lines 9-30. The variation between Ile, Leu. Met, and Val is clear and consistent between conservative substitutions and conservative/essential substitutions. In contrast, conservative substitutions between Ala, Ser, Thr, and Cys are inconsistent with conservative/essential substitutions between Thr and Lys. Moreover, conservative substitutions between Lys and Arg are inconsistent with conservative/essential substitutions between Lys and Thr. Thus, the possible substitutions of Lys and Thr that fall within the scope of the claim are unclear." Claim 11 further limits the particular non-native essential amino acids with which the sequence is modified. Applicants thank the Examiner for pointing out the difficulty in interpreting this claim as it was written. Applicants have amended this claim to specify that the essential amino acid residues "comprise isoleucine, lysine, threonine, tryptophan, methionine, leucine, valine or combinations thereof." Applicants point out that the claim has now been amended to specify the essential amino acids that were included in the list of conservative/essential substitutions on page 10, lines 29 and 30, but were not included in claim 11 prior to this amendment. Applicants submit that claim 11 as amended is definite, contains no new matter, and accordingly request that the rejection be withdrawn. The phrase "conservatively modified and conservatively substituted variants thereof" has been deleted from claim 28. Applicants also point out that they have amended claim 28 to delete the reference to the non-essential amino acids excluded from the claims at positions 56, 58, 59, 60, 61 and 62, since the claim only claims a

polypeptide modified to have essential amino acids at those positions. Thus, the claimed polypeptide would not be modified to have non-essential amino acids at these positions and the exclusion of non-essential amino acids is unnecessary.

18) Claim 12 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the term 'reduced' in claim 12 is a relative term which renders the claim indefinite. The term 'reduced' is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention." Applicants respectfully traverse this rejection. Example 7, which starts on page 62, line 27 of the specification, teaches the use of protease inhibition assays known to those of ordinary skill in the art that may be used to measure the inhibitory activity of a protein against chymotrypsin, subtilisin or elastase. Example 7, page 64, lines 3-5, teaches that "compared to wild type CI-2, the engineered proteins have reduced inhibitory activity against chymotrypsin, subtilisin, and elastase." Applicants have amended claim 12 to clarify that the reduced inhibitory activity is relative to the inhibitory activity of wild type CI-2. One of ordinary skill in the art would be able to perform the assay taught by Applicants in Example 7, and thus the scope of the invention claimed by Claim 12 is clear. In light of these arguments and amendments, Applicants respectfully request withdrawal of this rejection.

19) Claims 13, 14, and 16 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the term 'protein' in the instant claims does not have proper antecedent basis in the parent claim (10) which refers to polypeptides." Applicants thank the Examiner for this observation and have amended claims 13, 14 and 16 to refer to the term 'polypeptides,' for which proper antecedent basis exists. In light of these arguments and amendments, Applicants respectfully

request withdrawal of this rejection.

20) Claim 13 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that the term "comprising" should be recited as ---further comprising--- since the modifications cited are in addition to the positions in the parent claim (10). Applicants have amended claim 13 to insert the term "further" before the term "comprises." Applicants thank the Examiner for this suggestion and, in light of the amendments made, respectfully request withdrawal of this rejection.

21) Claim 14 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "Claim 14 is confusing for two reasons: 1) the further comprising language while not all the mutations in the list are options in the parent claim, and 2) the brackets around the apparent pairs of mutations." The Examiner suggested that Claim 14 be divided into two dependent claims to clearly claim the intended subject matter and suggested an acceptable format for noting the pairs of substitutions. Applicants have accordingly amended Claim 14 so that it reads "...comprising one of the following pairs of substitutions: T22C and V82C; or E23C and R8IC." Applicants have also added a new claim 96 that reads "...further comprising the pair of substitutions that is V53C and V7OC." Applicants thank the Examiner for this suggestion and, in light of the amendments made, respectfully request withdrawal of this rejection and allowance of new claim 96.

22) Claims 15-18 and 65-66 are rejected under 35 U SC. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the instant claims are confusing for four reasons: 1) in Claim 15 and Claim 65. the phrase "amino-terminal extension" is unclear, 2) in Claim 16, the phrase "nutritionally-enhancing polypeptide" is unclear, 3) in Claim 17 and Claim

66, the set of limitations on the amino-terminal extension are all unclear, and 4) in Claim 18, the location of the inclusion of residues 1 to 18 of Seq. ID NOs: 2 or 12 is confusing."

First, the Examiner states that "the phrase 'amino-terminal extension' is unclear because the term extending or extension implies using more of the same components, that is polypeptide; however, no such limitation is expressed in the claims or in the specification." Applicants respectfully traverse this rejection. The meaning of the term amino-terminal extension is clear from the specification, as is the fact that it refers to a polypeptide (which in some cases may constitute a protein) fused to the amino terminus end of the claimed polypeptide. For Example, page 67, lines 21-26 of the specification, states that "a construct was prepared that encoded a BHL3N dimer, with one BHL3N molecule fused at the amino terminus to the carboxy terminus of the other BHL3N molecule. ...The BHL3N polypeptide could also be fused at its animo terminus through genetic engineering methods known in the art, to another protein enriched in essential amino acids, such as high lysine hordothionin (Rao et al., Protein Engineering 7: 1485-1493, 1994). An amino terminal extension could also include a start signal, a transit sequence, a signal peptide, a fusion protein, a cleavable peptide, or an uncleaved peptide". Applicants submit that the specification does provide adequate definition of the amino terminal extensions that may be utilized with the claimed polypeptide.

Second, the Examiner states that "the phrase 'nutritionally-enhancing polypeptide' is unclear." To clarify what is meant, the claim has been amended to replace the phrase "nutritionally enhancing polypeptide" with the phrase "essential amino acid." This provides a definite description as to which nutritional components taught on page 9, lines 1-3 of the specification is being claimed. In light of this amendment, Applicants submit that Claim 16 points out and distinctly claims the subject matter which Applicants regard as the invention.

Third, the Examiner states that the terms which limit the amino-terminal extension in Claim 17 are wholly unclear. In response to the Examiners

rejections, Applicants have amended claims 17 and 66 to refer to a methionine, rather than a "start signal" or a "start methionine." In claim 17, the Examiner states that "the terms 'transit sequence', 'transit peptide', and 'signal peptide' have the same definition with respect to polypeptides (amino acid sequences which cause a translated protein to be transported into a membrane and optionally out of the compartment in which said protein was synthesized); thus, the inclusion of identical terms in a Markush-like group renders these terms unclear." Accordingly, Applicants have deleted the terms "transit sequence" and "transit peptide" from the claim. The Examiner also requested clarification as to whether the term "fusion protein" means the extension is a fusion protein itself or means the protein that is the extension is fused to the portion of the polypeptide that is related to Seq. ID No. 2 to make a 'fusion protein'. It is Applicants position that any polypeptide or protein combined with the polypeptide of claim 10 would constitute a polypeptide covered by claim 10. Accordingly, to expedite prosecution, Applicants have deleted the terms "fusion protein" and "CI-2 like polypeptide" from Claims 17. The Examiner similarly rejected the claims on the basis that "the terms 'cleavable peptide' and 'uncleaved peptide' are unclear since all peptides are cleavable and since an uncleaved peptide is merely a peptide." To expedite prosecution, Applicants have deleted the terms "cleavable peptide" and "uncleaved peptide" since, as stated above, such embodiments are covered by claim 10.

With respect to claim 18, the Examiner states that "the addition of residues similar to residues 1 to 18 of Seq. ID NOs: 2 or 12 is confusing because it implies that the additional residues are on the N-terminus; however, no such limitation is expressly stated in the claim." Applicant has amended claim 18 to replace the word "polypeptide" in line 1 with the words "amino-terminal extension", thereby clarifying that additional residues are on the N-terminus.

In light of these arguments and amendments, Applicants respectfully request withdrawal of this rejection and allowance of claims 15-18 and 65-66.

23) Claims 17, 19-25, 28, 32 and 87 were rejected under 35 U.S.C. 112,

second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that "the instant claims recite "CI-2 like" or "CI-2 derived" or "homologous to CI-2", none of which have clear definitions in the art or in the instant specification. Applicants note that Claims 17, 32, and 87 have been amended so that such terms are no longer used in those claims. Claims 19-25, and 28 have been amended to refer to the structural limitation of 30% sequence identity that is described in the definition of "CI-2 like" on page 8, lines 16-22, of the specification. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

24) Claims 19-25 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that "the particular modification of A75K or T is confusing since residue 75 is an asparagine (N) in Seq. ID No. 2. Applicants thank the Examiner for pointing out this typographical error in the claims and amend claims 19-25 to refer to N75K or T. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

25) Claims 19-25 were rejected under 35 U S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that "the Markush groups in Claim 19 and Claim 21 are unclear, particularly in view of the limitation of two or more modifications wherein the limitations are grouped. The Examiner suggests the writing out each possible modification, for example, H18A, H18I, H18L, etc. to ensure that each modification is considered separately to be within the set or two or more. Applicants thank the Examiner for this suggestion and have amended the claims accordingly. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

26) Claim 20 was rejected under 35 U S.C. 112, second paragraph, as

being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner has stated that "the claim should recite 'further comprise' (emphasis added) to indicate additional possible modifications. Additionally, the bracketing of the pairs of mutations is confusing (see above)." Applicants have amended the claim to state "further comprise" and to refer to the pairs of substitutions as suggested for claim 14 above. Applicants thank the Examiner for these suggestions, and in light of these amendments, respectfully request withdrawal of this rejection.

27) Claim 24 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection was similar to the ones for Claim 14 and Claim 20. Accordingly, Applicants have amended the claim to state "further comprise" where appropriate and to refer to the pairs of substitutions as suggested for claims 14 and 20 above. Applicants thank the Examiner for this suggestion and, in light of the amendments, respectfully request withdrawal of this rejection and allowance of amended claim 24 and new claim 97.

28) Claim 25 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the level to which the insert must be enriched with essential amino acids is unclear since 'enriched' is a relative term and no sequence is set forth for comparison." Applicants traverse this rejection. On page 68, lines 5-6 of the specification, Applicants state that "Peptides enriched in essential amino acids will be inserted into the active site loop region of the engineered proteins of the present study." One of ordinary skill in the art will understand that a peptide enriched with essential amino acids is one that will have the effect of further increasing the essential amino acid content of the modified protein. The level of enrichment may be small or large, however, in either case the essential amino acid content of the polypeptide of claim 21 will be further enhanced. Even a minor insertion of

one or two additional essential amino acids in the active site region would improve the essential amino acid content of a polypeptide already enhanced in essential amino acids. In light of these arguments Applicants submit that this rejection be withdrawn.

29) Claim 28 was rejected under 35 U.S.C 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner stated that "no Seq. ID No. is set forth in the instant claim for the corresponding positions cited." Applicants have amended the claim to clarify that the positions referred to correspond to the positions in Seq. ID No. 2. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

30) Claims 28, 29 and 62 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the terms 'conservatively modified' and 'conservatively substituted' are unclear. The Examiner states that "a definition is set forth in the instant specification on page 9. line 17; however; this definition has little limitation and also has undefined terms, like 'chemically similar'." Applicants point out that the term chemically similar is used in the definition of "conservatively modified variant" as it relates to amino acids on page 10 lines 4 to 13. However, the term chemically similar is not used to define "conservative substitutions" on page 10 lines 18-26. Six classes of conservative substitutions are defined. These are:

1) Alanine (A), Serine (S), Threonine (T), Cysteine (C);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Applicants submit that the term chemically similar is understandable to one of ordinary skill in the art and does not make the definition of "conservatively modified variant" indefinite. Notwithstanding, to expedite prosecution Applicants have amended claim 28 so that neither conservatively modified variant nor conservative substitution is used. Applicants have amended claims 29 and 62 so that only the term conservatively substituted variant is used, which term is clearly defined as described above. In lights of these arguments and amendments, Applicants respectfully request that this rejection be withdrawn.

31) Claims 31 and 58 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "in Claim 31, the phrase 'wherein the % sequence.. Weight of 4' is unclear as to whether or not the entire sequence or only any 23-mer is limited to be within 79% sequence identity. Applicants have amended this claim to clarify that the polypeptide must have more than 79% sequence identity to Seq. ID No. 20. The claim now reads, "A polypeptide with more than 79% sequence identity to the entire sequence of the polypeptide of Seq. ID No. 20, wherein the % sequence identity is determined by GAP analysis using Gap Weight of 12 and Length Weight of 4." In light of these amendments, Applicants respectfully request withdrawal of this rejection.

32) Claim 32 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that "the term 'immunologically reactive' is undefined." Applicants respectfully traverse this rejection.

On page 12 lines 7-9 of the specification Applicants state that "an indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide." Immunological reactivity can be used to define polypeptides that are substantially identical to Seq. ID No. 20 but not substantially identical to Seq. ID

No. 2. Applicants define what is meant by immunologically reactive on page 12, line 31, to page 13, line 6, of the specification, where they teach that "immunologically reactive conditions" are "conditions which allow an antibody, generated to a particular epitope, to bind to that epitope to a detectably greater degree (e.g., at least 2-fold over background) than the antibody binds to substantially all other epitopes. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols. See Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions." In light of these arguments, Applicants respectfully submit that this rejection be withdrawn.

33) Claim 62 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that "the phrase 'a wild-type CI-2 polypeptide' is confusing since only a single, native, wild-type CI-2 polypeptide is described in the instant specification, that is Seq. ID No. 2. The article 'a' indicates *any*, meaning more than one." Applicants have amended Claim 62 so that the proviso refers to specific sequences. These are Seq. ID. Nos. 2 and 4 and the wild type CI-2 homologs listed in Figure 2, which are Seq. ID Nos. 35-53. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

34) Claim 71 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. This rejection is similar to the one made for Claims 14, 20, and 24. Similar amendments have been made to claim 71. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

35) Claims 78-86 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the

subject matter which Applicants regard as the invention. The Examiner states that "it is confusing how a 23-mer polypeptide can correspond to positions 19-83 of another sequence, especially considering the broad sequence identity limitations." Applicants have deleted the phrase "of at least 23 amino acids in length". In light of these amendments, Applicants respectfully request withdrawal of this rejection.

36) Claim 86 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. This rejection is similar to the one above for Claims 14, 20, 24, and 71. Amendments similar to those made to claim 71 have been made to claim 86. In light of these amendments, Applicants respectfully request withdrawal of this rejection.

37) Claim 88 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that "the following genbank accession numbers, as found in Claim 88, cannot be defined: A01293, S37493, S33547, A01291, and A39547." Claim 88 has been deleted, thereby rendering this rejection moot. In addition, Applicants have amended the specification to refer to functional alternatives for such sequences, as described above in the amendments to the specification.

38) Claims 9-25, 28-31, and 54-89 were rejected under 35 U S.C. 112, first paragraph, written description, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states that "The instant claims are drawn to polypeptides having particular structural features, such as percent identity, percent mole composition, or modifications at particular positions of a defined structure, in the absence of *any* functional features."

The Examiner quotes the Court of Appeals for the Federal Circuit when it

stated that a "written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as be structure, formula [or] chemical name,' of the claimed subject matter sufficient to distinguish it from other materials." University of California v. Eli Lilly and Co., 1997 U.S. App. LEXIS 18221, at *23 quoting Fiers v. Revel, 25 LISPQ2d 1601, 1606 (Fed. Cir. 1993) (bracketed material in original). Applicant's submit that they have met the written description requirement as set forth by the Court of Appeals for the Federal Circuit.

A central issue in the case of *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997) involved the validity of a claim directed to a microorganism containing human insulin-encoding cDNA (claim 5 in the University of California patent number 4,652,525). As stated on page 1405 of *UC v. Eli Lilly*, "[T]he patent describes a method of obtaining this cDNA by means of a constructive example, Example 6. This example, however, provides only a general method for obtaining the human cDNA (it incorporates by reference the method used to obtain the rat cDNA) along with the amino acid sequences of human insulin A and B chains." Page 1405 also states "[B]ecause the '525 specification provides only a general method of producing human insulin cDNA and a description of the human insulin A and B chain amino acid sequences that cDNA encodes, it does not provide a written description of human insulin cDNA."

Unlike the situation in *UC v. Eli Lilly,* the instant specification provides specific methods and a complete written description for the polypeptides of the claimed invention. Methods of making and using the sequences of the invention are fully taught. Specific Seq. ID's and methods of further modifying these Seq. ID's are fully taught. In contrast to the '525 specification in *Lilly*, the instant specification sets forth specific parameters, e.g. number of substitutions, percent identity, mole percent and the like, all in reference to specific Seq. ID's, which describe and define the claimed invention.

The Examiner acknowledges that "the instant claims are drawn to polypeptides having particular structural features, such as percent identity, percent mole composition, or modifications at particular positions of a defined structure..." However, the Examiner rejects the claims on the basis that

"Applicants have described the claimed polypeptides in terms of structural and functional features, however, the claims do not recite the described function. Since the function of the claimed invention is extremely broad (any polypeptide can act as a nutritional supplement), the inclusion of such a functional clause in the instant claims will serve to identify the intended use of the products in the claims, which use is within the description in the instant specification. The intended use is required to define the claimed genus, as described, so that a polypeptide within scope of the structural limitations but having another intended use, such as enzymatic activity, would be considered a non-obvious species of the genus."

Applicants respectfully traverse this rejection. Neither the written description requirement nor the interpretation given to that requirement in *UC v. Eli Lilly* requires that claims be limited by both structure and function. Rather, the written description requirement, as described in *Lilly* supports that the breadth of description in the instant specification is adequate to support the claims. As stated in Lilly, "a description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus..." *Id.* at 1406. *Lilly* further cites In re Angstadt, 537 F.2d 498, 190 USPQ 214 (CCPA 1976), for the proposition that "applicants are *not* required to disclose *every* species encompassed by their claims even in an unpredictable art." *Id.* at 1406.

Applicants submit that description of a representative number of embodiments has been accomplished in the instant specification. Applicants have taught eight species of the CI-2 protein in Seq. ID Nos 6, 8, 10, 12, 14, 16, 18 and 20. Generic formulas, analogous to formulas used in chemical applications, are taught in Seq. ID Nos. 33 and 34. Throughout the specification Applicants teach how to obtain the claimed proteins, both through modification of nucleic acid sequences coding for the claimed proteins and through further modification of the proteins themselves. Without question, Applicants have taught those of ordinary skill in the art how to make and use the claimed modifications of wild type CI-2.

It can be inferred from a reading of *UC v. Eli Lilly* that claims drafted in terms of structural limitations are preferred over claims drafted in terms of functional limitations. Applicants have gone to great lengths to structurally define their claims as those useful in the practice of the present invention. For example, many of the claims, such as claims 10-25 are drawn to the specific embodiments taught in the specification. Other, such as claims 61-71, exclude from their scope modifications to the active site regions at positions 54-69, which by virtue of structural limitation excludes virtually all modifications made for altered enzymatic activity from the scope of the claims. For example, see Clausen et al. (WO 92/05239 A, IDS Paper No. 14) which focus on enzymatic modification of the active site region. Other claims have structural requirements such as requirements of a certain number of essential amino acid substitutions in combination with stabilizing non-native disulfide bonds that make it unlikely that such modified sequence would be suitable for use as enzymes. In summary, Applicants, by virtue of structural limitations already present in the claims have adequately defined the invention to a scope that bears a reasonable correlation with the scope of enablement.

The Examiner has suggested that Applicants amend the claims "to recite the intended use of the claims polypeptides, that is 'wherein the polypeptide is a nutritional supplement'." For the reasons stated above, Applicants believe that the scope of the claims is sufficient to define the invention as taught. Notwithstanding, Applicants have added the suggested functional limitation to claims 19, 21 and 28 in order to speed prosecution of these claims. Applicants also point out that although Applicants primarily teach use of the modified sequences as nutritional supplements, this is not mutually exclusive with effect on the enzymatic activity. As taught by Applicants on pages 48-49 of the specification, in the section Assays for Compounds that Modulate Protease Inhibitory Activity or Expression, and also on page 64, lines 3-5, polypeptides with reduced inhibitory activity are preferred for use in the present invention.

In light of these amendments, Applicants respectfully request withdrawal of this rejection.

39) Claim 32 was rejected under 35 U. S. C. 112, first paragraph, written description, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states that "the instant claim has no definite structural feature. Moreover, the function of being reactive with antibodies is wholly dependent on said structure. The instant specification fully describes polypeptide fragments of disclosed sequences which can interact with antibodies. However, the instant specification has not described all polypeptide fragments which would interact with the noted antibodies."

Applicants submit that the term CI-2 is defined in the specification and includes structural definition of the polypeptide. However, to expedite prosecution, Applicants have amended this claim to define the polypeptide as a polypeptide comprising conservatively substituted variants of Seq. ID No. 20. The term conservatively substituted variant is clearly defined as described in paragraph 30 above. As stated on page 12 lines 9-11 of the specification, "a polypeptide is substantially identical to a second polypeptide, for example, where the two polypeptides differ only by conservative substitution." Further, conservatively substituted variants of Seq. Id No. 20 were contemplated at the time of filing and do not constitute new matter. Page 17 line 3 to 25 discuss how to obtain variants, including conservatively substituted variants, of the present invention.

In light of these amendments, Applicants submit that the claim is to a polypeptide with definite structural features and that the rejection be withdrawn.

40) Claims 87-89 were rejected under 35 U.S.C. 112, first paragraph, written description, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states that "the instant claim is drawn to polypeptides which are homologous to CI-2." Applicants have amended claim 87 to refer to specific Seq. ID's as described above. Claims 88 and 89 have been

deleted. In light of these amendments, Applicants respectfully request that the rejection be withdrawn.

41) Claims 10-25, 28. 32, 56-58, and 61-89 were rejected under 35 U.S.C. 112, first paragraph, scope of enablement. The Examiner states that "the specification, while being enabling for polypeptides having a structure highly related to Seq. ID No.2 so that corresponding residues can be identified, does not reasonably provide enablement for polypeptides *not* having a structure highly related to Seq. ID No.2 so that corresponding residues *cannot* be identified." The Examiner further states that "the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention commensurate in scope with these claims. The instant claims are drawn to polypeptides comprising sequences which correspond to Seq. ID No.2 and are modified at particular corresponding positions. However, the ability to correspond Seq. ID No.2 with all polypeptide would require undue experimentation."

After citing the Wands factors, the Examiner states that "Polypeptides require a sufficient amount of relatedness to be aligned; such an alignment is required to identify corresponding positions. A large amount of experimentation would be required to determine corresponding positions in sequences which cannot easily be aligned to Seq. ID No. 2. Applicants present no guidance or working examples of such difficult alignments. In particular, it would be impossible to predict corresponding residues in the absence of sufficient structural relatedness. Thus, one of skill in the art would be required to perform undue experimentation to make the claimed products to the full extent of the claimed scope.

Applicants submit that an alignment of such residues in this manner is a procedure well known to those of ordinary skill in the art. The computer programs GAP and PileUp, each available from available from the University of Wisconsin Genetics Computer Group, may be used to determine an alignment of residues. One could easily utilize the methods taught by Applicant to create a polypeptide based on the sequence of wild type CI-2 (Seq. ID No. 2) and

determine the percent sequence identity with. Seq. ID No. 2. The Examiner has performed such an alignment and found a sequence by Kleber-Janke et al. with 43% sequence identity with Seq. ID No. 2. Such alignment programs are routinely used by those of ordinary skill in the art, are highly accurate, and do not require undue experimentation. Further, the claims as amended require that the claimed polypeptide sequences have at least 30% sequence identity with a reference sequence disclosed in the application. Thus, all claimed polypeptides will have a sufficient amount of relatedness to be aligned.

In light of these arguments and amendments made, Applicants respectfully request that the rejection be withdrawn.

Claim Rejections – 35 USC Section 102

42. Claim 9 was rejected under 35 U.S.C. 102(b) as being anticipated by Kleber-Janke et al. The Examiner states that "The instant claims are drawn to polypeptides with at least 30% sequence identity with Seq. ID No. 2 and having 15 mole % lysine. Kleber-Janke et al. teach a 68 amino acid peptide which is 43% identical to Seq. ID No. 2. Said peptide contains 10 lysine residues resulting in a mole percent of 14.7% (rounded up to 15%)."

As an initial matter, Applicants note that a prior application describing the subject matter of claim 9 as related to lysine was filed on November 1, 1996. (Novel High Lysine Proteins, Serial No. 08/740,682). Since this date is prior to the publication date of the Kleber-Janke reference, and since the present application claims priority to the above-referenced prior application, Applicants submit that the Kleber-Janke reference is not prior art and respectfully requests reconsideration and withdrawal of all rejections relying on the Kleber-Janke reference.

Although Applicants maintain that the Kleber-Janke reference is not a prior art reference, it is noted that, regardless, nothing in the Kleber Janke reference suggests the modification of Seq. ID No. 2 to increase its lysine content. Kleber Janke analyzed the gene expression in senescent barley leaves and identified the cDNA sequence cited by the Examiner. Kleber Janke did not alter the cDNA

sequence to have a mole percent of 14.7%. In light of these arguments, Applicants request reconsideration and withdrawal of this rejection.

43. Claims 10, 12, 13, 15-18, 28, and 87-89 were rejected under 35 U.S.C. 102(b) as being anticipated by Heim et al. The Examiner states that "The instant claims are drawn to polypeptides which are versions of Seq. ID No. 2 having seven or more essential amino acid residues at particular positions corresponding to Seq. ID No. 2 where Seq. ID No. 2 does not have essential amino acids. Said polypeptide also has a D74T modification with respect to Seq. ID No. 2. Said polypeptide is also homologous to known CI-2 polypeptides. Due to the confusing language of Claim 10 (see 112, second paragraph rejections above), polypeptides with changes other than at the identified positions meet the limitations of the instant claims.

The Examiner states that "Heim et al. teach a 212 amino acid protein having the following alterations with respect to Seq. ID No. 2 as aligned: S31T, L40T, Q41L, M59L, Y61L, D74T, I76V, Q78H and R8IH (the underlined modifications meet criteria of Claim 10). While these are not the only changes with respect to Seq. ID No. 2, the scope of the instant claims ("modified to contain") is unclear as noted above. Having a reduced inhibitory activity against various proteases is inherent in the polypeptide of Heim et al. to the extent that the 'reduced' activity is defined. The polypeptide of Heim et al. has ten amino acids prior to the corresponding amino acids which can be considered an N-terminal extension which is nutritionally enhancing and which is an uncleaved peptide and which at least one residue of Seq. ID No. 2 residues 1-18. The polypeptide of Heim et al. is homologous to known CI-2 polypeptides to the extent that homologous is defined (see above)."

Applicants respectfully contest this rejection. Heim is directed to a novel method for the production of a biologically active protein of interest by utilizing soluble yeast endoprotease (Kex2) and soluble yeast carboxypeptidase (Kex1). The Examiner has compared the sequence created by the Applicants to an Eglin C fusion protein with a Kex2/Kex1 site created by Heim. Heim has only modified this sequence to add the Kex2/Kex1 site. Heim has not modified the sequence

to contain seven or more non-native essential amino acids residues as required by claim 10. Thus, the Heim polypeptide falls outside of the scope of claim 10 and would therefore also fall outside of the scope of claims 12, 13 and 15-18. Additionally, claim 28 does not read on the Heim Eglin C fusion protein because the Heim Eglin C fusion protein is not a nutritional supplement. Claim 87, as amended, requires that the polypeptide be modified to contain a non-native disulfide bond, a feature not present in the Heim Eglin C fusion protein. Claims 88 and 89 have been deleted. In light of these arguments and amendments, Applicants submit that this rejection has been overcome.

44. Claims 19 and 21 were rejected under 35 U.S.C. 102(b) as being anticipated by Cordero et al. The Examiner states that "The instant claims are drawn to polypeptides having two or more particular modifications corresponding to Seq.. ID No. 2. Cordero et al. teach a 73 amino acid polypeptide with the following modifications with respect to Seq. ID No. 2: Q41K, I49V, and V79T (underlined modifications meet the criteria of Claim 21). While these are not the only changes with respect to Seq. ID No. 2, the scope of the instant claims ("CI-2 derived" and "corresponding to") is unclear as noted above."

Applicants point out that Cordero et. al. studied maize response to wounding and fungal infection. Cordero et. al. reported the isolation and characterization of cDNA and genomic clones for a gene encoding a maize proteinase inhibitor. Cordero et. al. did not perform any modification of the protein to increase its amino acid content or for any other purpose. Claims 19 and 21 as amended, specify that the claimed polypeptide is a nutritional supplement. The polypeptide identified by Cordero is not a nutritional supplement. In light of these arguments and amendments, Applicants submit that this rejection be withdrawn.

45. Claim 29 was rejected under 35 U.S.C. 102(b) as being anticipated by Clausen et al. (WO92/05239 A, IDS Paper No. 14). The Examiner states that "The instant claim is drawn to polypeptides related to Seq. ID Nos: 6, 8, 10, 12, 14, 16, 18, or 20. Clausen et al. teach an 83 amino acid polypeptide with high levels of relatedness to the above sequences (see attached alignments). The

Examiner notes that the terms "conservatively modified" and "conservatively substituted" are unclear.

Applicants have amended claim 29 to refer only to conservatively substituted variants as described in paragraph 30 above. Applicants submit that the term conservatively substituted variant is clear for the reasons described in paragraph 30 above. As such, it is clear that claim 29 does not read on the sequence of Clausen et. al., which contains several non-conservative modifications that distinguish it from Seq. ID Nos. 6, 8, 10, 12, 14, 16, 18 or 20 or conservatively substituted variants thereof. In light of these arguments and amendments, Applicants request that this rejection be withdrawn.

CONCLUSION

Applicants respectfully submit that in light of the foregoing amendments and remarks, Claims 9-25, 28-32 and 54-87 and new claims 96 and 97 are in condition for allowance. Further examination, reconsideration, and allowance of the claims are respectfully requested. If prosecution toward allowance could be furthered by a telephone call to the undersigned attorney for Applicants (515-254-2823), one is earnestly requested.

Respectfully submitted,
Rao, *et al.*

By _____
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APPENDIX "A"

Serial No. 09/311,689

Figure 1. Aragula Gururau Rao and Keith R. Roesler (Serial No. 09/311,689)
"PROTEINS WITH INCREASED LEVELS OF ESSENTIAL AMINO ACIDS"

SEQUENCES OF BHL PROTEINS

```
                                10        20        30        40        50        60        70        80
                                *         *         *         *         *         *         *         *
WT CI - 2 (native)  SSVEKKPEGVNTGAGDRHNLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLPVGTIVTMEYRIDRVRLFVDKLDNIAQVPRVG
WT CI-2                           MNLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLPVGTIVTMEYRIDRVRLFVDKLDNIAQVPRVG
BHL1                              MKLKTEWPELVGKSVEKAKKVILKDKPEAQIIVLPVGTKVTKEYKIDRVKLFVDKKDNIAQVPRVG
BHL2                              MKLKTEWPELVGKSVEKAKKVILKDKPEAQIIVLPVGTKVAKAYKIDKVKLFVDKKDNIAQVPRVG
BHL3                              MKLKTEWPELVGKSVEKAKKVILKDKPEAQIIVLPVGTKVGKHYKIDKVKLFVDKKDNIAQVPRVG
BHL3N             MKSVEKKPKGVKTGAGDKHKLKTEWPELVGKSVEKAKKVILKDKPEAQIIVLPVGTKVGKHYKIDKVKLFVDKKDNIAQVPRVG
BHL4                              MKLKTEWPELVGKSVEKAKKVILKDKPEAQIIVLPVGTKVTGEYKIDRVKLFVDKKDNIAQVPRVG
BHL5                             MAKMKTTWPELVGKTVEKAKKMIMKDKPEAKIMVLPVGTKVTGEWKMDRVKLWVDKKDKIAKTPKVG
BHL6                             MAKMKTTWPELVGKTVEKAKKMIMKDKPEAKIMVLPVGTKVTGEWKMDRVRLWVDKKDKIAKTPKVG
BHL8                             MAKMKCTWPELVGKTVEKAKKMIMKDKPEAKIMVLPVGTKVTGEWKMDRVRLWVDKKDKIAKTPKCG
BHLX                             XXXXKXXWPELVGKXVEXAKKXIXXDKPEAXIXVLPVGTXVXXXXXXDXVXLXVDKXDXXAXXPXXG
BHLXP                            XXXXKXXWPELVGKXXEXAKKXIXXDKPXAXIXVLPXGTXVXXXXXXXXVXLXXDKXDXXAXXPXXG
                                10        20        30        40        50        60        70        80
                                *         *         *         *         *         *         *         *
```

WT CI-2 (native) Seq ID #2
WT CI-2 Seq ID #4
BHL1 Seq ID #6
BHL2 Seq ID #8
BHL3 Seq ID #10
BHL3N Seq ID #12
BHL4 Seq ID #14
BHL5 Seq ID #16
BHL6 Seq ID #18
BHL8 Seq ID #20
BHLX Seq ID #33
BHLXP Seq ID #34

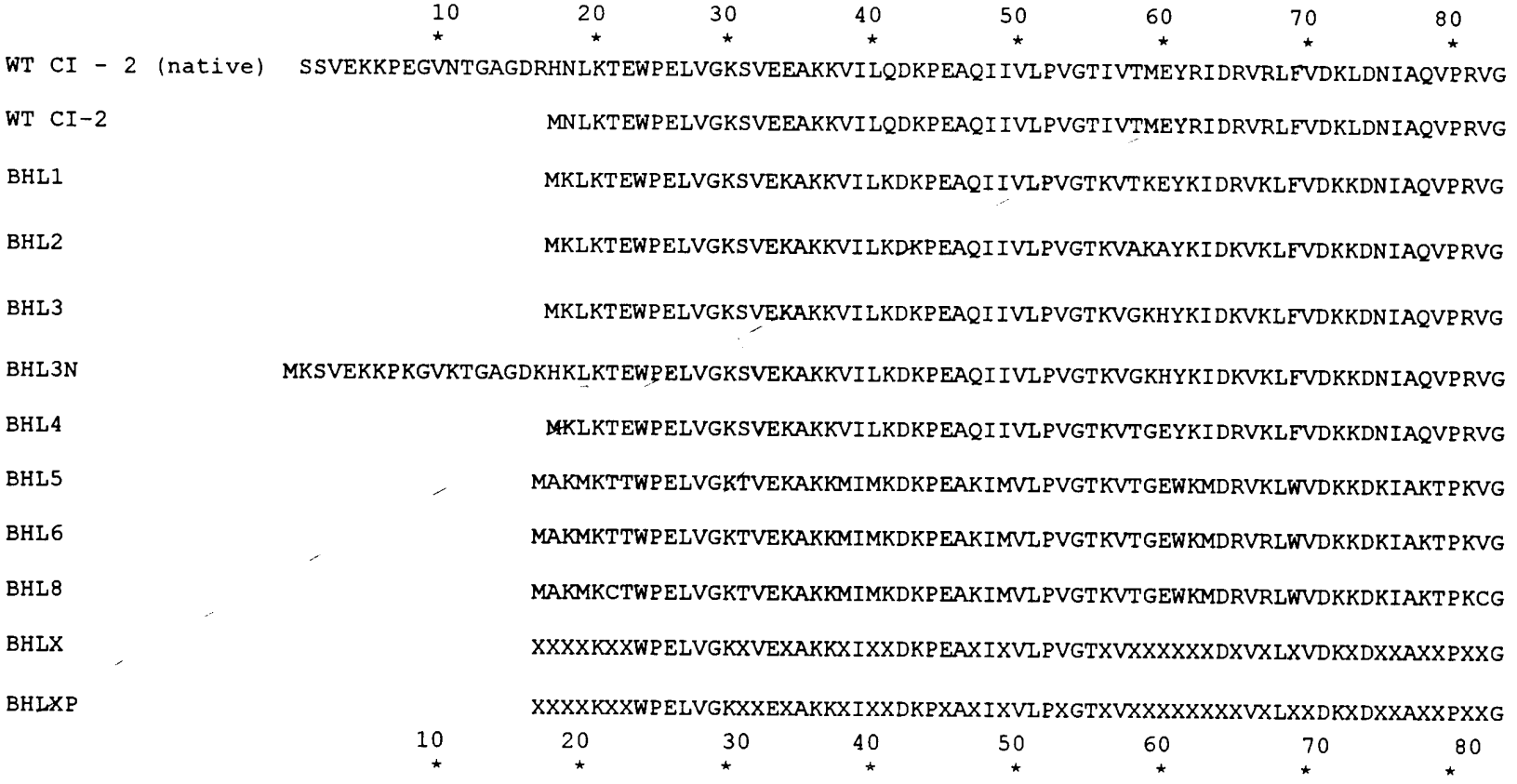
Figure 2. Aragula Gururau Rao and Keith R. Roesler (Serial No. 09/311,689)
"PROTEINS WITH INCREASED LEVELS OF ESSENTIAL AMINO ACIDS"

```
          20        30        40        50        60        70        80
           *         *         *         *         *         *         *
WT CI-2 MNLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLPV-GTIV-TMEYRIDRVRLFVDKLDNIAQVPRVG
Homologs
1         GAKTSWPEVVGMSAEKAKEIILRDKPNAQIEVIPV-DAMV-PLNFNPNRVFVLVHKATTVAZVSRVG
2         GAKTEWPELVGCTIKEAKEKIKADRPDLKVVIVPV-GSIV-TQEIDLNRVRVWVDKV---AKVPKIG
3         GAKTSWPEVVGLSVEDAKKVILKDKPDADIVVLPV-GSVV-TADYRPNRVRIFVDIV---AQTPHIG
4         --RTSWPELVGVSAEEARK-IKEEMPEAEIQVVPQ-DSFV-TADYKFQRVRLYVDESNKVVRAAPIG
5         AEKSSWPELVGEDGEEAVKIIQQENPSLDVILMPR-GQNWATLDCRPNRVRVFNDESGKVNSIPRIG
6         TRKTSWPELVGVTAEEAE-KIKEEMSGVEIQVVPP-GSFV-TADYKPQRVRLYVDESNKVTRTPGIG
7         PTKTSWPELVGVTAEQAETKIKEEMVDVQIQVSPH-DSFV-TADYNPKRVRKYVDESNKVTRTPSIG
8         VTKERWPELLGTPAKFAMQIIQKENPKLTNVQTVLNGTPV-TEDLRCNRVRLFVNVLDFVVQTPQVG
9         NKKETWPELIGVPAKFAREIIQKENSKLTNVPSVLNGSPV-TKDFRCERVRLFVNVLDFVVQIPRVG
10        VGKNTWPELCGARGEEAAATVETENPSVTAVIVPE-GSIV-TTDERCDRVRVWVDENGIVTRVPVIG
11        QGKSSWPQLVGSTGAAAKAVIERENPRVRAVIIKV-GSGA-TKDFRCDRVRVWVTERGIVARPPTIG
12        PGKSSWPHLVGVGGSVAKAIIERQNPNVKAVILEE-GTPV-TKDFRCNRVRIWVNKRGLVVSPPRIG
13        DGKLQWPELIGVPTKLAKEIIEKQNSLISNVHILLNGSPV-TMDFRCNRVRLFDDILGSVVQIPRVA
14        NGKLSWPELIGVPAHYAKGIIEKENSLITNVQILLNGSPV-TMDYRCNRVRLFDNILGDVVQIPRVA
15        KGKQFWPELIGVPALYAKGIIEKENPSITNIPILLNGSPV-TKDFRCDRVRLFVNILGDVVQIPRVT
16        VTKESWPELLGTPAKFAKQIIQKENPKLTNVETLLNGSAF-TEDLRCNRVRLFVNLLDIVVQTPKVG
17        EGKQMWPELIGVPTKLAKEIIEKENPSITNIPILLSGSPI-TLDYLCDRVRLFDNILGFVVQMPVVT
18        PGKQEWPELVGEYGYKAAAIIERENPNVRSIVKHE-RSGF-TKDFRCDRVWVVVDSTGVVVRTPRVT
19        VIFNSWSVLTGTNGDYAAVVIERENPTVNAAVILD-GSPV-TADFRCDRVRVFVDGNRIVVKTPKSG
```

WT CI-2=Seq.ID #4
Homologs 1=Seq. ID #35
Homologs 2=Seq. ID #36
Homologs 3=Seq. ID #37
Homologs 4=Seq. ID #38
Homologs 5=Seq. ID #39
Homologs 6=Seq. ID #40
Homologs 7=Seq. ID #41
Homologs 8=Seq. ID #42
Homologs 9=Seq. ID #43
Homologs 10=Seq. ID #44
Homologs 11=Seq. ID #45
Homologs 12=Seq. ID #46
Homologs 13=Seq. ID #47
Homologs 14=Seq. ID #48
Homologs 15=Seq. ID #49
Homologs 16=Seq. ID #50
Homologs 17=Seq. ID #51
Homologs 18=Seq. ID #52
Homologs 19=Seq. ID #53

Figure 1

SEQUENCES OF BHL PROTEINS



```
          20        30        40        50        60        70        80
           *         *         *         *         *         *         *
WT CI-2 MNLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLPV-GTIV-TMEYRIDRVRLFVDKLDNIAQVPRVG
Homologs
1         GAKTSWPEVVGMSAEKAKEIILRDKPNAQIEVIPV-DAMV-PLNFNPNRVFVLVHKATTVAZVSRVG
2         GAKTEWPELVGCTIKEAKEKIKADRPDLKVVIVPV-GSIV-TQEIDLNRVRVWVDKV---AKVPKIG
3         GAKTSWPEVVGLSVEDAKKVILKDKPDADIVVLPV-GSVV-TADYRPNRVRIFVDIV---AQTPHIG
4         --RTSWPELVGVSAEEARK-IKEEMPEAEIQVVPQ-DSFV-TADYKFQRVRLYVDESNKVVRAAPIG
5         AEKSSWPELVGEDGEEAVKIIQQENPSLDVILMPR-GQNWATLDCRPNRVRVFNDESGKVNSIPRIG
6         TRKTSWPELVGVTAEEAE-KIKEEMSGVEIQVVPP-GSFV-TADYKPQRVRLYVDESNKVTRTPGIG
7         PTKTSWPELVGVTAEQAETKIKEEMVDVQIQVSPH-DSFV-TADYNPKRVRKYVDESNKVTRTPSIG
8         VTKERWPELLGTPAKFAMQIIQKENPKLTNVQTVLNGTPV-TEDLRCNRVRLFVNVLDFVVQTPQVG
9         NKKETWPELIGVPAKFAREIIQKENSKLTNVPSVLNGSPV-TKDFRCERVRLFVNVLDFVVQIPRVG
10        VGKNTWPELCGARGEEAAATVETENPSVTAVIVPE-GSIV-TTDERCDRVRVWVDENGIVTRVPVIG
11        QGKSSWPQLVGSTGAAAKAVIERENPRVRAVIIKV-GSGA-TKDFRCDRVRVWVTERGIVARPPTIG
12        PGKSSWPHLVGVGGSVAKAIIERQNPNVKAVILEE-GTPV-TKDFRCNRVRIWVNKRGLVVSPPRIG
13        DGKLQWPELIGVPTKLAKEIIEKQNSLISNVHILLNGSPV-TMDFRCNRVRLFDDILGSVVQIPRVA
14        NGKLSWPELIGVPAHYAKGIIEKENSLITNVQILLNGSPV-TMDYRCNRVRLFDNILGDVVQIPRVA
15        KGKQFWPELIGVPALYAKGIIEKENPSITNIPILLNGSPV-TKDFRCDRVRLFVNILGDVVQIPRVT
16        VTKESWPELLGTPAKFAKQIIQKENPKLTNVETLLNGSAF-TEDLRCNRVRLFVNLLDIVVQTPKVG
17        EGKQMWPELIGVPTKLAKEIIEKENPSITNIPILLSGSPI-TLDYLCDRVRLFDNILGFVVQMPVVT
18        PGKQEWPELVGEYGYKAAAIIERENPNVRSIVKHE-RSGF-TKDFRCDRVWVVVDSTGVVVRTPRVT
19        VIFNSWSVLTGTNGDYAAVVIERENPTVNAAVILD-GSPV-TADFRCDRVRVFVDGNRIVVKTPKSG
----------------------------------------------------------------------------
```

APPENDIX "D"
Serial No. 09/311,689

1. Please replace the paragraph spanning lines 18 to 38 on page 5 of the specification with the following:

Figure 2 - CI-2-like sequences

1. Seq. ID No. 35, Hordeum vulgare (gi:68800) [(A01293)]
2. Seq. ID No. 36,Hordeum vulgare (Y08625)
3. Seq. ID No. 37, Zea mays (gi:475922)[(S37493)]
4. Seq. ID No. 38, Vicia faba (A21463)
5. Seq. ID No. 39, Cucurbita maxima (S55591, S12897)
6. Seq. ID No. 40, Canavalia lineata (JC2380)
7. Seq. ID No. 41, Vigna angularis (JX0089)
8. Seq. ID No. 42, Nicotiana tabacum (gi:19913)[(S33547)]
9. Seq. ID No. 43, Nicotiana sylvestris (A56555)
10. Seq. ID No. 44, Sambucus nigra (Z46949)
11. Seq. ID No. 45, Momordica charantia (JC2508)
12. Seq. ID No. 46, Cucurbita maxima (S12897)
13. Seq. ID No. 47, Solanum tuberosum (P01052, U30861)[(A01291)]
14. Seq. ID No. 48, Solanum tuberosum (U30861)
15. Seq. ID No. 49, Lycopersicon peruvianum (A39547)
16. Seq. ID No. 50, Lycopersicon esculentum (A32067, A24048)
17. Seq. ID No. 51, Lycopersicon esculentum (A24048)
18. Seq. ID No. 52, Amaranthus caudatus (S40496)
19. Seq. ID No. 53, Arabidopsis thalania (AC005770)

2. Please replace the paragraph spanning lines 21-22 on page 6 of the specification with the following:

Barley High Lysine 6 (BHL6) is coded for by the polypeptides of Seq. ID No. 18 [8] which is encoded for by the nucleic acid of Seq. ID No. 17.

3. Please replace the paragraph spanning lines 16 to 31 on page 8 of the specification with the following:

A "CI-2 like" polypeptide refers to a polypeptide of at least 23 consecutive amino acids of Seq. ID No. 2 or 4; or a polypeptide of at least 30% amino acid sequence identity with corresponding region of Seq. ID Nos. 2 or 4 or

20; or a CI-2-like polypeptide with modifications identified in CI-2; or a protease inhibitor with an active site loop typically between 53 and 70; or a CI-2 homologue modified to enhance its nutritional value by altering the amino acid residues at positions corresponding to those defined herein. The following organisms[(Genebank Accession Numbers)] may be modified according to the methods and figures in the specification Hordeum vulgare (Seq. ID No. 35)[(A01293)], Hordeum vulgare (Seq. ID No. 36)[(Y08625)], Zea mays (Seq. ID No. 37)[(S37493)], Vicia faba (Seq. ID No. 38)[(A21463)], Cucurbita maxima (Seq. ID No. 39)[(S55591, S12897)], Canavalia lineata (Seq. ID No. 40)[(CJ2380)], Vigna angularis (Seq. ID No. 41)[(JX0089)], Nicotiana tabacum (Seq. ID No. 42)[(S33547)], Nicotiana sylvestris (Seq. ID No. 43)[(A56555)], Sambucus nigra (Seq. ID No. 44)[(Z46949)], Momordica charantia (Seq. ID No. 45)[(JC2508)], Cucurbita maxima (Seq. ID No. 46)[(S12897)], Solanum tuberosum (Seq. ID No. 47)[(A01291,U30861)], Solanum tuberosum (Seq. ID No. 48)[(U30861)], Lycopersicon peruvianum (Seq. ID No. 49)[(A39547)], Lycopersicon esculentum (Seq. ID No. 50)[A32067, A24048)], Lycopersicon esculentum (Seq. ID No. 51)[(A24048)], Amaranthus caudatus (Seq. ID No. 52)[(S40496)], Arabidopsis thalania (Seq. ID No. 53)[(AC005770)].

4. Please replace the paragraph spanning lines 12 to 17 on page 12 of the specification with the following:

"Methods of alignment of sequences for comparison are well-known in the art. For purposes of defining the present invention, the BLAST 2.0 suite of programs using default parameters is used. Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997). Software for performing BLAST analyses is publicly available, e.g., such software may be retrieved from the National Center for Biotechnology Information over the Internet

<URL:http://www.ncbi.nlm.nih.gov>. [through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)]

5. Please replace the Abstract of the Disclosure on p. 76 of the specification with the following:

The invention provides isolated nucleic acids and their encoded polypeptides that are involved in enhancing the essential amino acid content of a plant. The polypeptide may be derived from a protease inhibitor, and more specifically, a chymotrypsin inhibitor. Chymotrypsin inhibitors that may be modified for use in the invention are present in many plant species, including barley. Optionally there is also a decrease in protease inhibitory activity of the polypeptide. The invention further provides recombinant expression cassettes, host cells, transgenic plants, and antibody compositions. The present invention provides methods and compositions relating to increasing essential amino acid content of plants for feed.

APPENDIX "C"

Serial No. 09/311,689

~~We claim:~~To the claims:

Please delete claims 1-8, 33-47, 49-53 and 88-95.

Please amend the claims as follows:

9. ~~(twice amended) A polypeptide~~(three times amended) A polypeptide with at least 30% sequence identity to the polypeptide of ~~SEQ ID NO:~~Seq. ID No. 2 and comprising greater than ~~50~~fifty amino acids in length and ~~altered to have the following composition:~~modified in order to have a composition selected from one of the following: at least 15-35 mole % lysine, ~~8-15~~at least 5-15 mole % methionine, ~~13-25~~at least 6-25 mole % threonine,~~6-12 mole % tryptophan, 12- 20 mole % isoleucine or combinations thereof;~~and at least 4-9 mole % tryptophan; wherein the % sequence identity is based on the entire sequence and is determined by BLAST 2.0 using default parameters.

10. (twice amended) A polypeptide~~(once amended) A polypeptide~~ comprising Seq. ~~ID. No. 2, or truncated versions thereof,~~ID No. 2 or 4 modified to contain ~~7~~seven or more non-native essential amino acid residues at positions corresponding to the positions in ~~Sequence ID.~~Seq. ID No. 2 selected from 1, 8, 11, 17, 18, 19, 20, 22, 23, 31, 34, 38, 40, 41, 47, 49, 56, 58, 59, 60, 61, 62, 63, 65, 67, 69, 73, 75, 76, 78, 79, 81, 82 or combinations thereof; and further provided that the polypeptide has at least 30% sequence identity to the polypeptide of Seq. ID No. 2 or 4, wherein the percent identity is determined by Blast 2.0 using default parameters.

11. ~~(once amended) The polypeptide of Claim 10 wherein the~~(twice amended) The polypeptide of Claim 10 wherein the essential amino acid residues comprise isoleucine, lysine, threonine, tryptophan, methionine, leucine, valine or combinations ~~or conservative substitutions thereof.~~thereof.

12. (once amended) The polypeptide of Claim 10 wherein the~~(no change) The polypeptide of Claim 10 wherein the~~ protein exhibits reduced inhibitory activity against chymotrypsin, subtilisin or elastase when compared with the inhibitory activity against chymotrypsin, subtilisin or elastase exhibited by wild type CI-2.

13. ~~(no change) The protein~~(once amended) The polypeptide of claim 10 wherein the polypeptide further comprises one or more of the following modifications: V32T; E45T; D64T; D74T; or A77T.

14. ~~(no change) The protein of claim 10 further~~(once amended) The polypeptide of claim 10 comprising one of the following ~~modifications: [T22C, V82C], [E23C, R81C] or [V53C, V70C].~~pairs of substitutions: T22C and V82C; or E23C and R81C.

15. (no change) The polypeptide of Claim 10 further comprising an amino-terminal extension.

16. (once amended) The polypeptide~~(no change) The protein~~ according to claim 15 wherein the amino terminal extension comprises ~~a nutritionally-enhancing polypeptide.~~essential amino acids.

~~18.~~17. ~~(once~~(twice amended)The polypeptide of Claim 15 wherein the amino-terminal extension is a ~~start signal, a transit sequence, a transit peptide, a signal peptide, a fusion protein, a cleavable peptide, a CI-2 like polypeptide or an uncleaved(once amended) The polypeptide~~methionine or a signal peptide.

18. (twice amended) The polypeptide of Claim 15 wherein the ~~polypeptide~~amino-terminal extension comprises at least ~~1~~one to about ~~18~~eighteen additional residues corresponding to amino acid residues 1 to 18 of Seq. ID No. 2 or 12.

19. (~~once~~(twice amended) ~~ACI-2 derived~~ polypeptide comprising two or more of the following modifications corresponding to positions in Seq. ID No. 2 selected from the group consisting of:

H18A, ~~I, L, V or M; N19K or T; L20M I, or V; E23T or K ; S31T or K; E34K or T; V38M I, or L; L40M I, or V; Q41K or T; Q47K or T; I49M I, L, or V; I56K or T; M59G; R62K or T; I63M, L, or V; R65K or T; R67K or T; F69W; L73K or T; A75K or T; Q78K or T; V79T or K; and R81K or T.~~H18I, H18L, H18V, H18M, N19K, N19T, L20M, L20I, L20V, E23T, E23K, S31T, S32K, E34K, E34T, V38M, V38I, V38L, L40M, L40I, L40V, Q41K, Q41T, Q47K, Q47T, I49M, I49I, I49L, I49V, I56K, I56T, M59G, R62K, R62T, I63M, I63L, I63V, R65K, R65T, R67K, R67T, F69W, L73K, L73T, N75K, N75T, Q78K, Q78T, V79T, V79K, R81K, and R81T; and further provided that the polypeptide is a nutritional supplement and has at least 30% sequence identity to the polypeptide of Seq. ID No. 2, wherein the percent identity is determined by Blast 2.0 using default parameters.

20. (~~no change~~)(once amended) The polypeptide according to claim 19 wherein the modifications further comprise one or more of the following ~~modifications: [E23C and R81C] or [T22C and V82C] or [V53C and V70C].~~pairs of substitutions: E23C and R81C; T22C and V82C; or V53C and V70C.

21. (~~once amended) A CI-2 derived~~(twice amended) A polypeptide comprising two or more of the following modifications corresponding to positions in Seq. ID No. 2 selected from the group consisting of:

~~H18A or M; N19K; L20M; T22C; E23T or C; S31T; E34K; V38M; L40M; Q41K; Q47K; I49M; I56K; M59G; R62K; I63M; R65K; R67K; F69W; L73K; A75K; Q78K; V79T; R81K or C; and V82C.~~H18A, H18M, N19K, L20M, T22C, E23T, E23C, S31T, E34K, V38M, L40M, Q41K, Q47K, I49M, I56K, M59G, R62K, I63M, R65K, R67K, F69W, L73K, N75K, Q78K, V79T, R81K, R81C, and V82C; and

further provided that the polypeptide is a nutritional supplement and has at least 30% sequence identity to the polypeptide of Seq. ID No. 2, wherein the percent identity is determined by Blast 2.0 using default parameters.

22. (once amended) The CI-2 derived(twice amended) The polypeptide of claim 21, further comprising substituting a tryptophan at position 61.

23. (no change) The polypeptide of claim 22, further comprising threonine at one or more of positions 32, 45, 53, 64 or 70.

24. (no change)(once amended) The polypeptide according toof claim 22 wherein the modifications comprise one or more of the following modifications: [E23C and R81C] or [T22C and V82C] or [V53C and V70C].pairs of substitutions: E23C and R81C; or T22C and V82C.

25. (no change) The polypeptide according to claim 22 further comprising an insert in the active site loop region that is enriched in essential amino acids for the purpose of nutritional enhancement.

28. (once(twice amended) ACI-2 derived polypeptide withcomprising three or more non-native essential amino acids replacing native amino acids at positions corresponding to Seq. ID No. 2 and selected from the group consisting of positions

1, 8, 11, 17, 18, 19, 20, 22, 23, 31, 32, 34, 38, 40, 41, 45, 47, 49, 56, 58, 59, 60, 61, 62, 63, 64, 65, 67, 69, 73, 74, 75, 76, 77, 78, 79, 81 and 82; and including conservatively modified and conservatively substituted variants thereof; and excluding V, P, W, S, E and R at position 56; S, K, R, P, E, V, Y, W, and A at position 58; R, Y, P,W, E, V, S, K, and A at position 59; Q, S,T, I, P, and K at position 60; V, E, R, P,excluding V and W at position 56; K, V and W at position 58; W, V and K at position 59; T, I and K at position 60; V and W at position 61 and E, Q, N, V, F, and Y position 62.V and F at position 62; and

further provided that the polypeptide is a nutritional supplement and has at least 30% sequence identity to the polypeptide of Seq. ID No. 2, wherein the percent identity is determined by Blast 2.0 using default parameters.

29. ~~(once~~(twice amended) A polypeptide comprising Seq. ID No. 6, 8, 10, 12, 14, 16, 18, 20 or conservatively~~modified or conservatively~~ substituted variants thereof.

~~31.~~30. ~~(once~~(twice amended) A polypeptide comprising at least ~~23~~twenty three contiguous amino acids of ~~SEQ.~~Seq. ID Nos. 6, 8, 10, 12, 14, 16, 18 or 20.

31. ~~(once~~(twice amended) A polypeptide~~comprising at least 23 contiguous amino acids~~ with more than 79% sequence identity~~,~~ to the polypeptide of Seq. ID No. 20, wherein the ~~%~~percent sequence identity~~is based on~~ the 23 ~~contiguous amino acids sequence and~~ is determined by GAP analysis using Gap Weight of 12 and Length Weight of 4.

32. (once amended) A~~CI-2 derived~~ polypeptide comprising a conservatively substituted variant of the polypeptide of Seq. ID No. 20 that is immunologically reactive with antibodies against the ~~protein~~polypeptide of Seq. ID No. 20 and not ~~SEQ~~the polypeptide of Seq. ID No. 2.

54. ~~(once~~(twice amended) The polypeptide of claim 9, wherein the polypeptide is ~~altered to have 8-15~~modified in order to have a composition of at least 5-15 mole % methionine.

55. ~~(once~~(twice amended) The polypeptide of claim 9, wherein the polypeptide is ~~altered to have 13-25~~modified in order to have a composition of at least 6-25 mole % threonine.

56. ~~(new)~~(once amended) The polypeptide of claim 10 wherein the non-native essential amino acid residues comprise lysine and the positions correspond to the positions in ~~SEQ. Id.~~Seq. ID No. 2 selected from 1, 8, 11, 17, 19, 34, 41, 56, 59, 62, 65, 67, 73 or combinations thereof.

57. ~~(new)~~(no change) Food or feed comprising the polypeptide of claim 30.

58. ~~(new)~~(no change) Food or feed comprising the polypeptide of claim 31.

59. ~~(once~~(twice amended) A polypeptide with at least 60% sequence identity to the polypeptide of ~~SEQ ID NO:~~Seq. ID No. 2 comprising greater than ~~50~~fifty amino acids in length and comprising ~~about 15 mole % or more lysine based on the total number of~~more than seven lysine amino acid residues.

60. ~~(once~~(twice amended) The polypeptide of Claim 59, further comprising ~~about 20 mole % or more lysine based on the total number of~~more than ten lysine amino acid residues.

61. ~~(new)~~(once amended) A polypeptide selected from the group consisting of:

(a) a polypeptide comprising ~~Sequence ID NOS.~~Seq. ID Nos. 6, 8, 10, 12, 14, 16, 18 or 20; and

(b) a polypeptide comprising any one of ~~Sequence ID NOS.~~Seq. ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20, wherein said polypeptide has been modified to contain ~~an~~more than four non-native essential amino acids at~~4 to about 34~~ positions~~in a range~~ corresponding to ~~Sequence~~Seq. ID No. 2 positions 19-53 and ~~63-83~~.70-83, and wherein said modified polypeptide has at least 30% sequence identity to the polypeptide of Seq. ID No. 4 or 20, the percent sequence identity determined by Blast 2.0 using default parameters.

62. ~~(new)~~(once amended) A polypeptide comprising a conservatively~~modified or conservatively~~ substituted variant of the polypeptide of claim 61, with the proviso that the polypeptide is not ~~a wild type CI-2 polypeptide.~~ Seq. ID No. 2, 4 or 35-53.

63. ~~(new)~~(no change) The polypeptide of claim 61, wherein the essential amino acid is isoleucine, lysine, tryptophan, methionine, threonine, or mixtures thereof.

64. ~~(new)~~(no change) The polypeptide of claim 61, wherein the essential amino acid is lysine.

65. ~~(new)~~(no change) The polypeptide of claim 61, further comprising an amino terminal extension.

66. ~~(new)~~(once amended) The polypeptide of claim 65, wherein the amino terminal extension comprises a~~start~~ methionine.

67. ~~(new)~~(no change) The polypeptide of claim 65, wherein the amino terminal extension comprises essential amino acids.

68. ~~(new)~~(no change) The polypeptide of claim 61, further comprising at least one non-native cysteine.

69. ~~(new)~~(once amended) The polypeptide of claim 68, wherein the non-native cysteine is at one or more positions corresponding to ~~Sequence~~Seq. ID No. 2 positions 23, 81, 22, 82, 53 or 70.

70. ~~(new)~~(no change) The polypeptide of claim 61, further comprising at least two non-native cysteines.

71. ~~(new)~~(once amended) The polypeptide of claim 70, wherein the non-native cysteines are at ~~positions (T22C, V82C) or (E23C, R81C).~~one or more of the following pairs of positions: T22C and V82C; or E23C and R81C.

72. ~~(new) A polypeptide of at least 23 amino acids in length~~(once amended) A polypeptide comprising any one of ~~Sequence ID NOS.~~Seq. ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20, wherein said polypeptide has been modified to contain at least one non-native disulfide bond and ~~at least 4 to about 34~~more than four non-native essential amino acids in positions corresponding to ~~Sequence~~Seq. ID No. 2 positions ~~19-83.~~19-83, and wherein said modified polypeptide has at least 30% sequence identity to the polypeptide of Seq. ID No. 4 or 20, the percent sequence identity determined by Blast 2.0 using default parameters.

73. ~~(new)~~(no change) The polypeptide of claim 72 wherein the non-native essential amino acids are lysine.

74. ~~(new)~~(once amended) A polypeptide comprising any one of ~~Sequence ID NOS.~~Seq. ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20, wherein the polypeptide has been modified to contain a~~more than seven~~ non-native essential amino ~~acid at 6 to about 34~~acids in positions~~in a range~~ corresponding to ~~Sequence~~Seq. ID No. 2 positions 19-53 and 63-83 and wherein said modified polypeptide has at least 30% sequence identity to the polypeptide of Seq. ID No. 4 or 20, the percent sequence identity determined by Blast 2.0 using default parameters.

75.(~~new~~)no change) The polypeptide of claim 74 wherein the non-native essential amino acids are lysine.

76.~~(new)~~(once amended) A polypeptide~~of at least 23 amino acids in length~~ comprising any one of ~~Sequence ID NOS.~~Seq. ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20, wherein the ~~sequence is~~polypeptide has been modified to

contain at least ~~11~~eleven non- native essential amino acids in positions corresponding to ~~Sequence~~Seq. ID No. 2 positions 19-83, and wherein said modified polypeptide has at least 30% sequence identity to the polypeptide of Seq. ID No. 4 or 20, the percent sequence identity determined by Blast 2.0 using default parameters.

77. ~~(new)~~(no change) The polypeptide of claim 76 wherein the non-native essential amino acids are lysine.

78. ~~(new) A polypeptide of at least 23 amino acids in length and having at least 64% identity sequence of amino acid residues corresponding to positions 19-83 in Sequence ID No. 2 and comprising~~(once amended) A polypeptide having at least 60% sequence identity to the polypeptide of Seq. ID No. 4 and modified to contain a non-native disulfide ~~bond with at least one cysteineresidue in at least one position~~ bond. ~~corresponding to positions 19-83 in Sequence ID No. 2.~~

79. ~~(new)~~(once amended) A polypeptide~~of at least 23 amino acids in length~~ having at least 60% identity to ~~a sequence of amino acid residues in Sequence ID No. 6 and comprising~~the polypeptide of Seq. ID No. 6 and modified to contain a non-native disulfide ~~bond with at least one cysteine residue in at least one position corresponding to positions 19-83 in~~ bond. ~~Sequence ID No. 2.~~

80. ~~(new)~~(once amended) The polypeptide of claim 79, having at least ~~74% dentity to the sequence of amino acid residues.~~70% sequence identity to the polypeptide of Seq. ID No. 6. ~~(new) A polypeptide of at least 23 amino acids in length and having at least~~

81. ~~57% identity to a sequence of amino acid residues in Sequence ID No. 8, and further comprising~~(once amended) A polypeptide having at least 60%

sequence identity to the polypeptide of Seq. ID No. 8 and modified to contain a non-native disulfide ~~bond with at least one cysteine residue in at least one position corresponding to positions 19-83 in~~ bond. ~~Sequence ID No. 2.~~

82.~~(new)~~(once amended) The polypeptide of claim 81, having at least ~~67% identity to the sequence of amino acid residues.~~70% sequence identity to the polypeptide of Seq. ID No. 8.

83.~~(new) A polypeptide of at least 23 amino acids in length and having at least 57% identity to a sequence of amino acid residues in Sequence ID No. 10 and comprising~~ (once amended) A polypeptide having at least 60% sequence identity to the polypeptide of Seq. ID No. 10 and modified to comprise a non-native disulfide ~~bond with at least one cysteine residue in at least one position corresponding to positions 19-83 in~~ bond. ~~Sequence ID No. 2.~~

84.~~(new)~~(once amended) The polypeptide of claim 83, having at least ~~67% identity to a sequence of amino acid residues.~~70% sequence identity to the polypeptide of Seq. ID No. 10.

85.85.~~(new)~~(once amended) The polypeptide of claim 84, wherein the non-native disulfide bond is formed with a cysteine residue~~is substituted~~ at one or more ~~positions corresponding to Sequence ID NO. 2~~of positions 23, 81, 22, 82, 53 or 70.

86.~~(new) The polypeptide of claim~~(once amended) The polypeptide of claim 85, wherein the non-native disulfide bond is ~~between positions corresponding to 81).~~formed between cysteine residues substituted at one or more of the following pairs of positions: 22 and 82; or 23 and 81.

87.87.(once amended) A polypeptide comprising any one of Seq. ID Nos. 24, 26, 28, 30, 32 or 35-53, wherein the polypeptide is modified to have a non-native disulfide ~~(new) A polypeptide homologous to CI-2, wherein said~~

~~polypeptide is at least 20 amino acids in length and is modified to have~~ ~~least~~bond and more than seven non-native essential amino acid residues. ~~residues and comprising a non-native disulfide bond.~~

96. (new) The polypeptide of claim 10 further comprising the pair of substitutions that is V53C and V70C.

97. (new) The polypeptide of claim 22, further comprising the pair of substitutions that is V53C and V70C.

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Rapid Gastric Fluid Digestion and Biochemical Characterization of Engineered Proteins Enriched in Essential Amino Acids

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The barley high lysine (BHL) proteins are nutritionally enhanced derivatives of barley chymotrypsin inhibitor-2 (CI-2). A compactly folded new CI-2 derivative, BHL9, was engineered with the highest content of threonine, tryptophan, and isoleucine yet achieved in this protein family (15.1, 9.4, and 12.1 wt %, respectively). BHL9 had an unfolding midpoint of 5.5 M guanidinium chloride, significantly greater than values for wild type (3.9 M) or for the previously most stable BHL protein, BHL8 (3.6 M). BHL9 and all other derivatives were digested within 15 s in simulated gastric fluid (SGF), suggesting nutritional availability upon ingestion. Denaturation of the proteins in SGF minus pepsin was revealed by changes in their fluorescence emission spectra and/or far UV circular dichroism spectra. The proteins lack homology to known allergens. Significantly, the BHL8 and BHL9 proteins were stable to proteases at pH 7.5 or 8.0, attesting to their potential for high expression in plants.

Keywords: *Gastric fluid; intestinal fluid; protein digestion; nutritional proteins; protein engineering; protein stability*

INTRODUCTION

The protein of major cereal crops does not contain an optimum amino acid balance for animal or human nutrition. This suboptimal balance limits livestock growth and health (*1*). One approach to improving the nutritional value of crops is to overexpress proteins containing a high content of the limiting amino acids. With this aim in mind, several proteins derived from barley chymotrypsin inhibitor-2 (CI-2) were previously engineered for an increased essential amino acid content (*2*, *3*). The most promising of these engineered proteins, BHL8 (barley high lysine protein 8) was substantially enriched in lysine, methionine, tryptophan, and threonine, retained no inhibitory activity against digestive enzymes of monogastric animals, and contained an engineered disulfide bond that increased stability against proteolysis by trypsin and chymotrypsin. Such in vitro protein stability was a reassuring indication of structural integrity but also raised the question of whether BHL8 would be nutritionally available when eaten. One goal of the present study was therefore to assess the digestibility of the BHL proteins by simulated gastric fluid and simulated intestinal fluid.

Another goal of the present study was to assess the versatility of the CI-2 scaffold to meet diverse nutritional needs. Even a promising protein such as BHL8 is not optimal for solving amino acid requirements in all cases. As compared to poultry, for example, swine have a higher requirement for tryptophan and a lower requirement for the sulfur amino acids (*1*). Furthermore, the relative amino acid requirements vary with the age of the animal. In addition, the relative content of essential amino acids varies between conventional maize and high oil maize, and between maize and the other cereals. For example, maize protein has only approximately half the tryptophan content of protein from the other cereals (*4*). Finally, the relative price and commercial availability of free amino acids for use as feed supplements can influence the amino acid goals. It is apparent, therefore, that a single engineered protein would not be optimal for meeting amino acid goals in all situations. A flexible protein platform that could be tailored for a specific amino acid content, depending on the intended use, would be helpful. To this end, we have explored the flexibility of the CI-2 protein as a platform for nutritional enhancement of maize and other crops.

We here report the design of another disulfide stabilized CI-2 derivative, BHL9, that has greater in vitro protein stability and a higher content of threonine, tryptophan, and isoleucine, in comparison with BHL8. In addition, we demonstrate that all members of the BHL protein family are readily digested by enzymes of the gastrointestinal tract of monogastric animals.

MATERIALS AND METHODS

BHL9 Expression and Purification. A synthetic gene encoding the BHL9 protein was prepared by Midland Certified Reagent Company (Midland, TX) and ligated into the *Nco*I and *Hind*III restriction sites of expression vector pET 28 (Novagen, Madison, WI). BHL9 was expressed in *Escherichia coli* BL21 DE3 in 2× YT media. Cultures were grown at 37 °C to an $OD_{600\ nm}$ of 0.6 to 0.8, then induced with 1 mM IPTG and transferred to 16 °C for growth overnight. The protein was purified by cation exchange and gel permeation chromatography as reported previously (*3*), except that the gel permeation chromatography was done in 50 mM Tris-HCl, 150 mM NaCl, pH 8.6. N-terminal amino acid sequencing and MALDI mass spectrometry analyses of purified BHL9 were performed by the Iowa State University Protein Facility (Ames, IA).

SDS–PAGE and Immunoblot Analysis. SDS–PAGE was performed with precast Tris-Tricine 16.5% gels from BioRad Laboratories (Hercules, CA) as per manufacturer's instructions. Proteins were visualized by Coomassie brilliant

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blue. For the immunoblots of Figures 6 and 9, protein from SDS–PAGE was blotted to Immobilin-P PVDF membrane from Millipore (Bedford, MA) with a Trans-Blot SD semidry transfer cell (BioRad Laboratories, Hercules, CA). Blots were blocked in 3% bovine serum albumin 15 min, then probed with a 1:1000 dilution of rabbit anti-sera against either wild-type CI-2 (Figure 9 blot and wild-type blot of Figure 6) or against a 1:1 mixture of BHL6 (*2*) and BHL8 (BHL8 blot of Figure 6). Proteins were visualized by probing with a goat anti-rabbit alkaline phosphatase conjugate according to the method of the manufacturer (Promega Biotec, Madison, WI).

BHL Protein Quantitation. A molar extinction coefficient at 280 nm of 23 000 M^{-1} cm^{-1} was used for BHL9 quantitation, and previously determined coefficients were used for the other BHL and wild-type proteins (*2*, *3*).

Protease Inhibition Assays. Apparent K_i values for BHL9 were determined with the proteases and method described previously (*3*).

Trypsin and Chymotrypsin Digests. Bovine pancreatic trypsin and chymotrypsin from Sigma Chemical Company (St. Louis, MO) were quantitated by absorbance at 280 nm using an extinction coefficient (1% protein, 1-cm path length) of 16.0 for trypsin and 20.4 for chymotrypsin. Digests were done at 37 °C for 4 h in 100 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0. Three micrograms of BHL8 or BHL9 were incubated with or without 0.3 μg of protease in a 15 μL volume. Reactions were stopped by adding an equal volume of BioRad 2× Tris-Tricine SDS sample buffer containing 200 mM dithiothreitol and 6 mM PMSF, followed by boiling 5 min and SDS–PAGE.

Simulated Gastric Fluid Digests. SGF contained 3.2 mg/mL porcine pepsin (Sigma Chemical Company, St. Louis, MO) in 34 mM NaCl, 0.7% HCl, pH 1.2 (*5*). Incubations were done at 37 °C with 15 μL of SGF per 3 μg of target protein. This gave a ratio of 16:1 (wt/wt) pepsin/target protein, which is similar to the 18.8:1 ratio used previously in a study of allergenic and nonallergenic proteins (*6*). At the desired times, 15 μL aliquots of the incubation mix were transferred to a stop solution composed of 15 μL of BioRad 2× Tris-Tricine SDS sample buffer containing 200 mM dithiothreitol, plus 1 μL of 1.5 M Tris-HCl, pH 8.8. For the 0 time controls of Figure 5, SGF and substrate protein were added directly to the stop solution without prior incubation. Digestion was assessed by SDS–PAGE and Coomassie brilliant blue staining. To preincubate BHL8 or WT CI-2 with a maize protein matrix (Figure 6 experiment), whole kernel flour from opaque-2 maize was extracted 4 h at 25 °C in a 10:1 vol/wt ratio of 50 mM Tris-HCl, 100 mM KCl, 5% glycerol, pH 8.0. Maize protein was quantitated by the method of Bradford (*7*) using the reagent from BioRad Laboratories (Hercules, CA) with bovine gamma globulin as standard. A 20:1 wt/wt ratio of maize protein/BHL8 or WT CI-2 was incubated 30 min at 37 °C before proceeding with the SGF digests.

Determination of Initial BHL8 Peptic Cleavage Sites. BHL8 (60 μg) was digested 15 min at 37 °C in 34 mM NaCl, 0.7% HCl, pH 1.2, with a 1:50 (wt/wt) ratio of pepsin/BHL8. The peptic fragments were purified by reversed phase chromatography with a PepRPC HR 5/5 column (Amersham-Pharmacia; Piscataway, NJ). Elution was performed in 0.1% TFA with a 30 mL, 10–45% acetonitrile gradient. N-terminal sequencing and MALDI mass spectrometry were performed by the Iowa State University Protein Facility.

Simulated Intestinal Fluid Digests. SIF is defined as 50 mM potassium phosphate, pH 7.5, containing 10 mg/mL pancreatin (*5*). However, the porcine pancreatin used in the present study (Sigma Chemical Company, St. Louis, MO catalog no. P-7545) was 8× United States Pharmacopeia specifications, and therefore, 1.25 mg/mL, rather than 10 mg/mL, was used. Incubations were done at 37 °C, and at the desired times, aliquots containing 3 μg of BHL or wild-type protein in 15 μL of SIF were transferred to a stop solution composed of 16 μL of BioRad 2× Tris-Tricine SDS sample buffer containing 200 mM dithiothreitol and 6 mM PMSF, and immediately boiled one minute. The 0 time controls were done by adding SIF and substrate proteins directly to stop solution, without prior incubation.

Digestion of BHL8 Peptic Fragment by Trypsin or Simulated Intestinal Fluid. To generate the BHL8 peptic fragment, BHL8 (20 μg) was digested with 0.4 μg of porcine pepsin in 40 μL of 34 mM NaCl, 0.7% HCl, pH 1.2, at 37 °C for 15 min. The reaction was stopped by adding 40 μL of 1 M Tris-HCl, pH 8.0. Twenty microliters of the 80 μL total volume was transferred to SDS sample buffer and represented lane 2 of Figure 7. Another 20 μL of the peptic fragment was further digested by addition of 0.5 μg of trypsin and incubation at 37 °C 30 s, and the reaction was stopped by addition of an equal volume of BioRad 2× Tris-Tricine SDS sample buffer containing 200 mM dithiothreitol and 5 mM PMSF. This digest represented lane 3 of Figure 7. An additional 20 μL aliquot of the peptic fragment was further digested by incubation with SIF at 25 °C 30 s, and the reaction was stopped as done with trypsin. This digest represented lane 4 of Figure 7.

Purification and Quantitation of CI-2 Homologues from Mature Maize Kernels. Twenty grams of opaque-2 maize whole kernel flour was extracted in 100 mL of 70% ethanol 18 h at 25 °C. Centrifugation was done at 17000*g* for 15 min, and the supernatant was saved. The pellet was back extracted with an additional 70 mL of 70% ethanol, and centrifugation was repeated. The two supernatants were combined and poured through one layer of Miracloth (Calbiochem-Novabiochem, La Jolla, CA). Ethanol was then added to 90%, and stirring was done at 25 °C, 40 min. Centrifugation was done at 17000*g* 15 min. The pellet was resuspended in 20 mL of 50 mM Tris-HCl, pH 8.0, and recentrifuged to remove insoluble material. The supernatant, representing the 70–90% ethanol fraction, was applied to a protease-agarose (Sigma Chemical Company, St. Louis, Mo.) column containing 2 units of Protease VIII A from *Bacillus licheniformis*. The column was washed with 10 column volumes of 50 mM Tris-HCl, pH 8.0, then eluted with 5 column volumes of 100 mM glycine, pH 2.0. Neutralization was done with an equal volume of 1 M Tris-HCl, pH 8.0. Buffer was changed to 10 mM sodium phosphate, pH 7.0, with a disposable PD-10 column from Amersham-Pharmacia, and the protein was concentrated by ultrafiltration with Centricon-3 concentrators (Millipore; Bedford, MA). A total of 164 μg of affinity purified CI-2 homologues resulted, as determined by absorbance at 205 nm using an extinction coefficient (1 mg/mL) of 27 + 120 (A_{280}/A_{205}) (*8*). This quantitation method is known to be relatively independent of amino acid composition of the protein. Assuming the 20 g of flour was 10% protein, and assuming a 10% recovery during extraction, purification and concentration, the CI-2 homologues collectively comprised about 0.08% of total kernel protein.

Circular Dichroism and Fluorescence. Far UV CD and fluorescence emission spectra were determined as described previously (*2*) except that protein concentrations of 5 μM, rather than 2 μM, were used for fluorescence analyses. For the samples in simulated gastric fluid minus pepsin, CD and fluorescence analyses were begun 5 min and 30 s, respectively, after addition of the protein to the pH 1.2 buffer.

Homology Search of Allergen Database. A TFASTA search (*9*) of the Genetics Computer Group, Inc. (GCG, Madison, WI) Allergens database was made using a gap creation penalty of 16 and a gap extension penalty of 2.

RESULTS

Design of BHL9. The BHL9 composition and amino acid sequence are presented in Table 1 and Figure 1, in comparison with WT CI-2 and other BHL proteins. The intent with BHL9 was to make changes in the BHL8 composition favorable for swine nutrition, without disrupting protein folding and stability. Thus, the two cysteine substitutions at positions 22 and 82 that formed a stabilizing disulfide bond in BHL8 (*2*) were retained in BHL9. Threonine, tryptophan, and isoleucine were increased in BHL9, at the expense of some methionine and lysine. The methionine reduction was considered acceptable because this amino acid is far less limiting

Table 1. Amino Acid Composition of Wild-Type and Engineered CI-2

	WT CI-2			BHL8			BHL9			average proteins[a]	maize endosperm proteins[b]
AA	#	mol%	wt%	#	mol%	wt%	#	mol%	wt%	mol%	wt%
Ala	3	4.6	3.1	4	6.0	4.0	4	6.0	4.1	8.3	8.1
Arg	4	6.1	8.0	2	3.0	3.9	2	3.0	4.0	5.7	3.8
Asn	2	3.0	3.0	0	0	0	0	0	0	4.4	6.2 (Asn+Asp)
Asp	4	6.1	6.1	4	6.0	6.0	4	6.0	6.1	5.3	
Cys	0	0	0	2	3.0	2.7	2	3.0	2.8	1.7	1.8
Gln	3	4.6	5.0	0	0	0	0	0	0	4.0	21.3 (Gln+Glu)
Glu	6	9.1	10.1	4	6.0	6.6	3	4.5	5.1	6.2	
Gly	3	4.6	2.6	4	6.0	3.4	4	6.0	3.5	7.2	3.2
His	0	0	0	0	0	0	0	0	0	2.2	2.8
Ile	6	9.1	9.0	3	4.5	4.4	8	11.9	12.1	5.2	3.8
Leu	6	9.1	9.0	3	4.5	4.4	2	3.0	3.0	9.0	14.3
Lys	6	9.1	10.1	16	23.9	26.3	10	14.9	16.8	5.7	2.0
Met	2	3.0	3.4	6	9.0	10.1	2	3.0	3.4	2.4	2.8
Phe	1	1.5	1.9	0	0	0	0	0	0	3.9	5.3
Pro	4	6.1	5.3	4	6.0	5.2	4	6.0	5.3	5.1	9.7
Ser	1	1.5	1.2	0	0	0	0	0	0	6.9	5.2
Thr	3	4.6	4.1	5	7.5	6.7	11	16.4	15.1	5.8	3.5
Trp	1	1.5	2.4	3	4.5	6.9	4	6.0	9.4	1.3	0.5
Tyr	1	1.5	2.1	0	0	0	0	0	0	3.2	5.3
Val	10	15.2	13.5	7	10.5	9.2	7	10.5	9.4	6.6	4.7
Total	66	100.3	99.9	67	100.4	99.8	67	100.2	100.1	100.1	104.3

[a] Average of 1021 unrelated proteins (*28*). [b] Glover and Mertz (*21*).

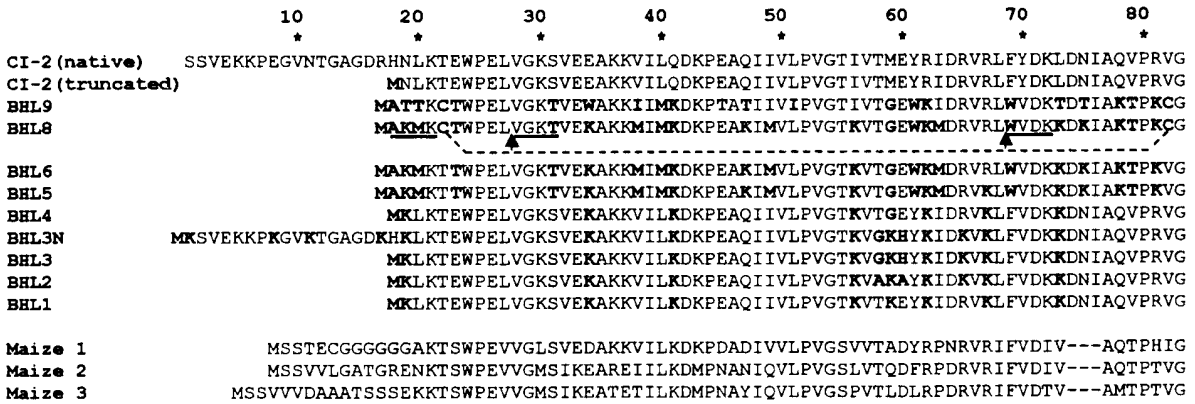

Figure 1. Amino acid sequences of engineered and wild-type barley CI-2 and homologous sequences from maize endosperm. Mutated residues are in bold. Arrows indicate the initial peptic cleavage sites of BHL8. Underlined residues were determined by N-terminal sequencing of BHL8 peptic fragments. Dashed lines represent the disulfide bond between Cys22 and Cys82 in BHL8 and BHL9. Native CI-2 sequence is from Williamson et al. (*27*).

for swine than for poultry. Lysine is the first limiting amino acid for swine fed a typical corn/soybean diet (*1*), but lysine is much less expensive as a feed supplement than threonine, tryptophan, or isoleucine. Therefore, lysine substitutions at several positions in BHL8 were resubstituted with threonine, tryptophan, or isoleucine in BHL9. In choosing the BHL9 substitutions, careful consideration was given to an alignment of homologous sequences of CI-2 from diverse plant species (*2*). For example, a tryptophan substitution was made at position 34 of BHL9, even though this is not a conservative change in comparison with BHL8 or WT CI-2, because several homologous sequences had aromatic residues at this position. Similarly, numerous homologues had isoleucine at position 51, and threonine (or serine) at position 45, suggesting that these substitutions in BHL9 were acceptable. The lysine, threonine, tryptophan, sulfur amino acid, and isoleucine contents of the BHL proteins are presented in Table 1. These values are much higher than those of proteins in general, or of maize endosperm protein in particular.

Biochemical Characterization of BHL9. BHL9 was initially expressed in *E. coli* at 37 °C and purified with the same method as used previously for the other BHL proteins. However, much lower yields were consistently observed with BHL9 than with any of the other proteins (<50 μg of BHL9 versus several milligrams of the other proteins per liter of culture). Growth overnight at 16 °C after induction increased BHL9 expression severalfold, but yields were still much lower than for the other BHL proteins at 37 °C. Amino terminal sequencing of purified BHL9 gave the sequence "Ala–Lys–Met–Lys", revealing that the start methionine was not retained in BHL9 (Figure 1), in agreement with previous observations for some of the other BHL proteins.

Protease inhibitory activities of BHL9 were determined with pancreatic chymotrypsin, trypsin, and elastase, and also with subtilisin Carlsberg from *B. licheniformis*. No inhibitory activities against chymotrypsin, trypsin, or elastase were observed, consistent with previous observations for BHL8 and the closely

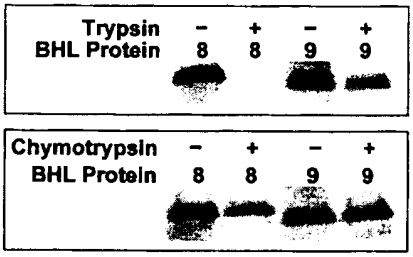

Figure 2. Relative resistance of BHL9 and BHL8 to digestion by trypsin and chymotrypsin. Incubations were done at 37 °C for 4 h without protease or with 1:10 (wt:wt) protease/substrate.

related proteins BHL5 and BHL6 (*2*). However, BHL9 was an effective inhibitor of subtilisin, with an apparent K_i value of 11.6 ± 2.1 nM. This is similar to the WT value but is very different from the BHL8 value of 415 ± 3 nM (*2*). Thus, one or more of the BHL9 amino acid substitutions resulted in a significant restoration of inhibitory activity against this bacterial protease. The WT isoleucines that were restored in BHL9 at positions 56 and 63 are both located in the reactive site loop region and perhaps contributed to this restoration of inhibitory activity.

The BHL9 thermodynamic stability was determined by equilibrium unfolding experiments with guanidinium chloride, as reported previously (*2*). An unfolding midpoint of 5.5 M GdmCl was determined for BHL9, significantly greater than the previously determined values of 3.9 M for wild type CI-2 and 3.6 M for BHL8, the most thermodynamically stable BHL protein previously characterized.

The structural integrity of BHL9 was also examined by incubation with the proteases trypsin and chymotrypsin (Figure 2) and compared with the most proteolytically stable BHL protein identified previously, BHL8. Despite having many potential tryptic cleavage sites (Lys and Arg), BHL9 was relatively resistant to digestion by trypsin, suggesting that BHL9 was compactly folded. In contrast, no BHL8 protein survived the trypsin incubation intact, but this may be due in part to the higher content of lysine (and therefore of potential tryptic cleavage sites) of BHL8, rather than reflecting a difference in compactness of folding. Stability against digestion by chymotrypsin, which cleaves at aromatic residues, was also examined (Figure 2). Partial digestion of BHL8, but not BHL9, was evident. Thus, BHL9 appeared to be more resistant to digestion by chymotrypsin, despite having one additional potential chymotryptic cleavage site at position 34 that was not present in BHL8. The four tryptophans in BHL9 are located in a β-sheet (Trp 24 and Trp 69), an α-helix (Trp 34) and the reactive site loop (Trp 61). The fact that these residues were relatively inaccessible to chymotrypsin suggested that BHL9 was compactly folded.

Changes in Protein Conformation in Simulated Gastric Fluid Minus Pepsin. Wild-type CI-2, BHL9, BHL8, and BHL6 (identical to BHL8 except for the disulfide bond) were analyzed by far UV circular dichroism spectroscopy at pH 7 and also at pH 1.2 in simulated gastric fluid minus pepsin (Figure 3). At pH 7, the BHL9 spectrum was very similar to that of its parent protein BHL8, suggesting similar secondary structures. Both showed a minimum at 207 nm, with a slightly less negative ellipticity for BHL9. Major changes in the

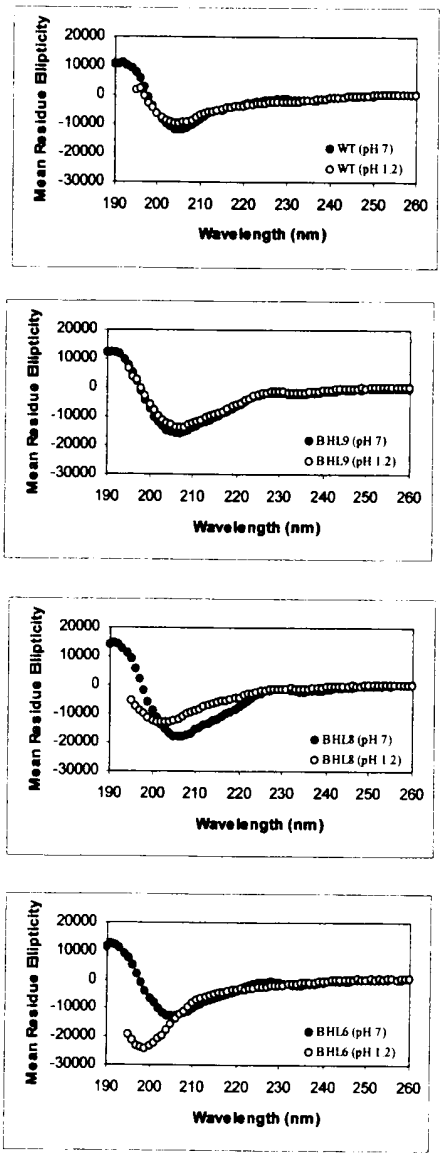

Figure 3. Far UV circular dichroism spectra of wild-type and engineered CI-2. Analyses were done with a protein concentration of 20 μM in either 10 mM sodium phosphate, pH 7, or in simulated gastric fluid minus pepsin (pH 1.2).

BHL6 and BHL8 spectra were observed at pH 1.2 as compared with pH 7, suggesting substantial changes in the secondary structure of the two proteins at low pH. In contrast, only minor differences were observed for the wild type and BHL9 spectra at these two pH values, indicating greater retention of secondary structure in these proteins at low pH. The BHL9 spectrum even appeared to retain the minimum at 234 nm at pH 1.2. This minimum was previously attributed to tryptophan 24 of wild-type CI-2 (*10*).

The fluorescence emission spectra of the proteins were also examined at neutral pH and in simulated gastric fluid minus pepsin (Figure 4). Immediate changes in fluorescence intensity were observed for all of the proteins upon addition to the SGF buffer, with an increase in intensity noted for wild type CI-2 and

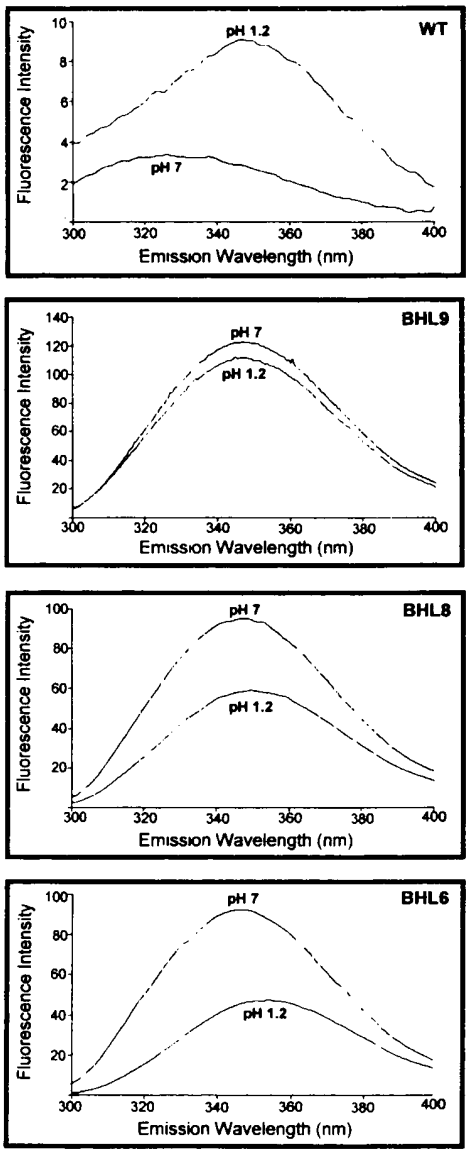

Figure 4. Fluorescence emission spectra of wild-type and engineered CI-2. Excitation wavelength was 280 nm. Analyses were done with a protein concentration of 5 μM in either 10 mM sodium phosphate, pH 7, or in simulated gastric fluid minus pepsin (pH 1.2).

decreases for the other three proteins. Fluorescence intensities were lowest, as expected, in the wild-type protein, which contained only a single tryptophan. Red shifts in the peak emission wavelength at low pH were also observed with all of the proteins except for BHL9, suggesting greater exposure of tryptophan(s) at low pH. Taken together, the results of Figures 3 and 4 suggested that all of the proteins underwent conformational changes at low pH, with the least change occurring in the BHL9 protein.

Digestion by Simulated Gastric Fluid. Digestion of the engineered and wild-type proteins by simulated gastric fluid (SGF) was examined (Figure 5). All of the engineered and wild type proteins were digested by SGF within 15 s, including BHL9, the protein showing the

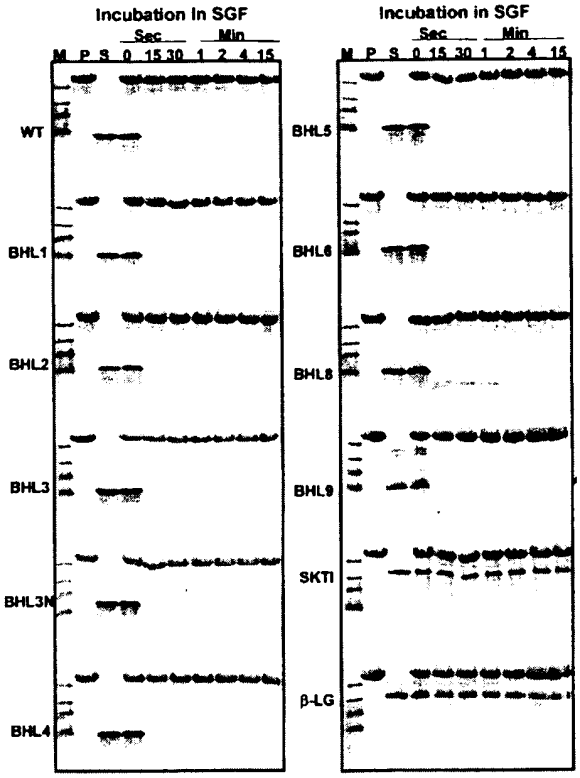

Figure 5. Digestion of wild-type and engineered CI-2 by simulated gastric fluid (SGF). SDS–PAGE and Coomassie brilliant blue staining were used to assess digestion. Lane M, molecular mass markers of 26.6, 17.0, 14.4, and 6.5 kDa. A 3.5 kDa marker is also barely visible in some gels. Lane P, pepsin (no substrate protein); lane S, substrate protein (no pepsin); other lanes, substrate protein incubated at 37 °C in SGF for the indicated times. SKTI = soybean Kunitz trypsin inhibitor. β-LG = beta subunit of lactoglobulin from bovine milk.

least change in CD or fluorescence emission spectra at low pH. In contrast, the soybean Kunitz trypsin inhibitor and the beta subunit of bovine milk lactoglobulin were completely stable in SGF, in agreement with previous results (*6*). For some of the engineered proteins, the peptic fragments were large enough to detect by SDS–PAGE. For example, two peptic fragments of BHL5, BHL6, and BHL8 were visible at 15 s. Only the smaller of the two was detected at 1 min, and this fragment gradually diminished during the time course, but was still barely visible in BHL8 at 15 min. For other proteins such as BHL4, BHL9, and wild-type CI-2, the peptic fragments were too small to be detected by this gel system, even at 15 s.

It is possible, though unlikely, that a maize protein could bind to the engineered proteins and stabilize them against pepsin digestion. For example, perhaps a very acidic maize protein could bind to the basic BHL proteins and directly block peptic cleavage sites or indirectly block them by preventing unfolding of the BHL proteins. This hypothesis was tested by incubation of BHL8 or wild-type CI-2 with a maize protein extract. However, the two proteins were still digested by SGF within 15 s (Figure 6).

The susceptibility of the most persistent BHL8 peptic fragment to further digestion by trypsin or simulated

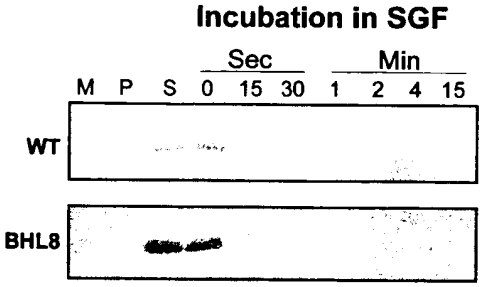

Figure 6. Digestion of wild-type CI-2 and BHL8 by simulated gastric fluid (SGF), following incubation in a corn protein matrix. Pure BHL8 or CI-2 were incubated 30 min in a 20-fold excess of corn protein. SGF digests were then done as in Figure 5, except that immunoblot analysis of gels was done, rather than Coomassie brilliant blue staining.

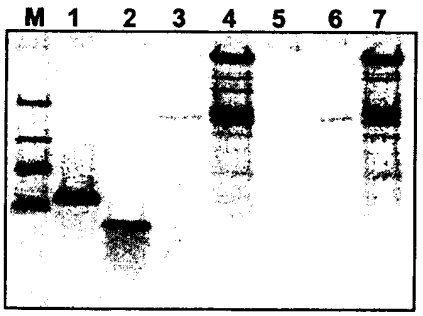

Figure 7. Digestion of BHL8 peptic fragment by trypsin and by simulated intestinal fluid (SIF). SDS–PAGE and Coomassie brilliant blue staining were used to assess digestion. Lane M, molecular mass markers of 26.6, 17.0, 14.4, and 6.5 kDa; lane 1, BHL8 (5 μg); lane 2, BHL8 peptic fragment resulting from digestion of 5 μg of BHL8; lane 3, BHL8 peptic fragment (same quantity as in lane 2) incubated 30 s with trypsin; lane 4, BHL8 peptic fragment (same quantity as in lane 2) incubated 30 s with SIF; lane 5, pepsin only; lane 6, trypsin only; lane 7, SIF only.

intestinal fluid (SIF) was also examined (Figure 7). The BHL8 peptic fragment was digested within 30 s by either trypsin or SIF. Thus, the BHL8 peptic fragment was much more easily digested by trypsin than was the intact BHL8 protein, which was still detectable in 1:10 trypsin after 2 h (*2*).

To determine the initial BHL8 peptic cleavage sites, digestion with a dilute pepsin concentration was performed, and the resulting BHL8 peptic fragments were purified by reversed phase chromatography. Three protein peaks were purified and subjected to N-terminal sequencing and MALDI analysis (Figure 1). One peak had a sequence of "Val–Gly–Lys–Thr" and a mass of 4.7 kDa. Another peak had a double sequence of "Ala, Trp–Lys, Val–Met, Asp–Lys" and a mass of 2.9 kDa, while the third peak represented residual undigested BHL8. The sequences and masses of the peptic fragments were identical to those expected for pepsin cleavage of BHL8 after Leu-27 and Leu-68, and were consistent with the protein containing a disulfide bond, and not retaining the start Met. An examination of the 3-D structure of the folded protein suggested that Leu-27 and Leu-68 are unlikely to be accessible to pepsin, as the side chains of these residues are buried ($\approx$ 0% solvent accessibility). The extremely low pH of SGF apparently denatured the protein, exposing these peptic cleavage sites, consistent with the changes in the BHL8

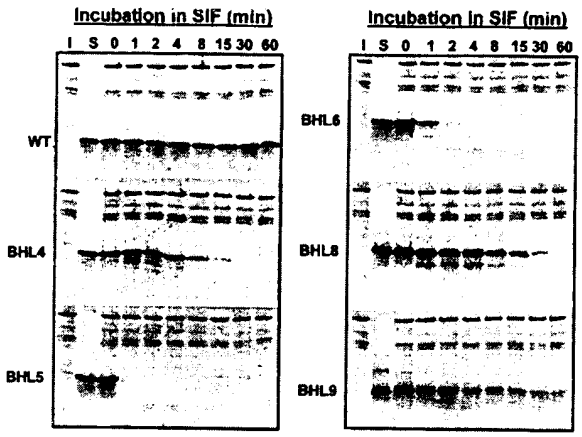

Figure 8. Digestion of wild-type and engineered CI-2 by simulated intestinal fluid (SIF). SDS–PAGE and Coomassie brilliant blue staining were used to assess digestion. Lane I, SIF only; lane S, substrate protein only; other lanes, substrate protein incubated at 37 °C in SIF for the indicated times.

Table 2. Comparison of Thermodynamic and Proteolytic Stability of Wild-Type and Engineered CI-2

protein	unfolding midpoint [GdmCl] (M)[a]	survival time in SIF (min)	survival time in SGF (s)
WT CI-2	3.9	60 (no digestion evident)	<15
BHL9	5.5	60 (some digestion evident)	<15
BHL8	3.6	30	<15
BHL4	2.6	15	<15
BHL6	1.8	2	<15
BHL5	1.3	<1	<15

[a] Value for BHL4 was from Roesler and Rao (*3*). Values for other proteins (except BHL9) were from Roesler and Rao (*2*).

CD spectra and fluorescence emission spectra at low pH noted previously (Figures 3 and 4).

Digestion by Simulated Intestinal Fluid. On the basis of the results of Figure 5, it seemed unlikely that the BHL proteins would survive passage through the stomach to reach the intestine intact. Nevertheless, it was interesting to look at the digestibility of wild type CI-2 and several of the intact BHL proteins by simulated intestinal fluid (SIF) (Figure 8). Wild-type CI-2 was the most stable protein in SIF, with no digestion evident after even 60 min in this potent protease cocktail. BHL9 was the most stable of the engineered proteins, with some intact BHL9 detected at 60 min. Substantial digestion of BHL9 was also evident at 60 min, however, unlike for wild type CI-2. BHL8 and BHL4 were also relatively resistant to digestion by SIF, with intact protein evident at 30 and 15 min, respectively. In contrast, BHL6 and BHL5 were much less resistant to digestion, surviving only 2 min or <1 min, respectively. The thermodynamic stability of the proteins correlated well with their stability in SIF at pH 7.5, but not with their stability in SGF at pH 1.2 (Table 2). The only exception to the correlation was wild type CI-2, which was thermodynamically less stable than BHL9, yet more stable in SIF. This result may be explained by the fact that wild-type CI-2 is a potent inhibitor of chymotrypsin and elastase (*11*), both components of SIF, but BHL9 is not an inhibitor of these proteases.

Assessment of Allergenicity Risk. According to the FAO/WHO report on the evaluation of allergenicity (Evaluation of Allergenicity of Genetically Modified Foods, http://www.fao.org/WAICENT/FAOINFO/ECONOMICS/ESN/gm/biotec-e.htm), the methods include (i) in vitro digestibility in SGF, (ii) sequence homology searches, and (iii) specific serum screening.

In the present paper, we have used the first two methods in the evaluation of BHL proteins. The SGF digests presented in Figure 5 were helpful not only for predicting nutritional availability but also for predicting the risk of food allergenicity. It has been suggested that food allergens tend to be stable in SGF, while nonallergens tend to be quickly digested (*6*). The BHL proteins behaved like nonallergens, being digested within 15 s, while the Kunitz trypsin inhibitor and β-lactoglobulin, which are both known food allergens (*6*), were stable.

Another means of predicting the risk of food allergenicity is to search the protein of interest for sequence identity with known allergens. A protein containing six consecutive amino acids that are identical to the sequence of a known allergen or having 35% identity in an 80-amino acid stretch is considered to be at risk of being an allergen. Therefore, a search of the GCG allergen database was made with the BHL8 and BHL9 amino acid sequences. No matches of greater than four consecutive amino acids were identified nor were overall sequence identities of >35% found. The serum of individuals allergic to barley would be tested for binding of IgE to CI-2 and derivatives only if successful in planta expression of the proteins is achieved.

Overexpressing the engineered proteins in maize would also appear less likely to introduce a new allergen if similar, naturally occurring proteins were already present in maize kernels. To determine the presence of CI-2 homologues in maize, a search with the wild type CI-2 sequence was made of the Pioneer Hi-Bred maize embryo, endosperm, and whole kernel EST databases. EST sequences encoding three CI-2 homologues from maize endosperm were identified (Figure 1). Homologue 1 was also previously determined to be expressed in maize germinating embryos (*12*) and leaves (*13*). The N-terminal regions of the homologues were nonconserved and appeared unstructured, as determined previously with the barley CI-2 N-terminal region (*14*). Over the 65-amino acid region from positions 19 to 83, homologue 1 had amino acid sequence identities of 62, 52, and 51% with WT CI-2, BHL8, and BHL9, respectively. The corresponding values for homologue 2 were 54, 46, and 46%, and the values for homologue 3 were 52, 46, and 45%. To approximately quantitate the levels of the CI-2 homologues in mature opaque-2 maize kernels, a purification method was developed that included ethanol fractionation and affinity chromatography with a protease agarose column (Figure 9). The polypeptide(s) represented by the most prominent band in lane 1 of Figure 9 ran near the 7.5 kDa molecular mass marker, bound to the protease, and cross-reacted with polyclonal antibodies against barley CI-2. These observations were all consistent with expectations for CI-2 homologues. Using assumptions described in Materials and Methods, we estimated that the CI-2 homologues collectively comprised between 0.01 and 0.1% of the total protein in mature maize kernels.

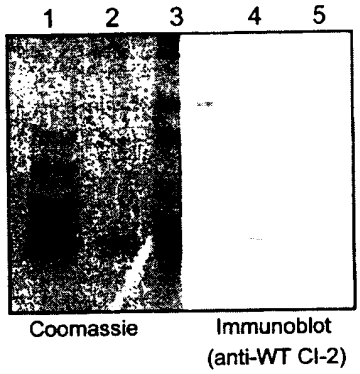

Figure 9. Purification of CI-2 homologues from mature maize kernels. Lanes 1 and 4, protein purified by 70 to 90% ethanol fractionation; lanes 2 and 5, protein affinity purified with a protease-agarose column. Lane 3 (split for both staining and blotting), prestained molecular mass markers of 7.5, 18.4, 32.5, 45.7, 78, 132, and 216 kDa.

DISCUSSION

Nutritional Availability of Engineered Proteins. Our studies suggest that the BHL proteins will be nutritionally available when eaten by humans or monogastric animals. Both the engineered and wild-type proteins were digested by SGF within 15 s, consistent with a previous observation that the CI-2 inhibitory activity against chymotrypsin was eliminated by a 5 min incubation with pepsin at pH 2.0 (*15*). Considering that there is a lag period of 10 to 70 min following ingestion before any solid food is emptied from the human stomach (*16*), it seems unlikely that the BHL proteins, or wild-type CI-2, would reach the intestine intact following ingestion. Furthermore, the unstructured peptic fragments of such lysine rich proteins would contain numerous tryptic cleavage sites, allowing rapid further digestion by trypsin in the intestine, as demonstrated here for the most stable BHL8 peptic fragment. In the unlikely event that some of the BHL proteins did reach the intestine intact, they would probably still be nutritionally available, as both BHL8 and BHL9 were completely or substantially digested within 60 min in SIF and were also devoid of inhibitory activity against intestinal proteases. Unlike the BHL proteins, wild-type CI-2 was completely stable in SIF. Therefore, if any intact wild-type protein reached the intestine, it could pass through the animal unused and inhibit digestive proteases. Previous feeding studies suggested that even this was an unlikely scenario. Rats and mice fed uncooked HiProly barley, a naturally occurring high lysine line with elevated CI-2 levels, gained weight faster than those fed wild-type barley (*17*). Thus, CI-2 did not appear to be a major antinutritional factor, consistent with its lability in SGF evident here.

Prospects for Nutritional Improvement of Plants. Previous studies suggested that proteins with a high degree of stability against proteolysis in vitro were more likely to accumulate to high levels in plants. For example, high methionine mutants of soybean glycinin that were susceptible to proteolysis in vitro did not accumulate in transgenic tobacco, in contrast to the proteolytically more stable wild-type glycinin (*18*). Legumin modified with a methionine rich C-terminal extension was proteolytically unstable in vitro and also did not accumulate in transgenic plants (*19*). In addi-

tion, a high methionine phaseolin mutant that was malfolded (and therefore probably more susceptible to proteases) did not accumulate in transgenic tobacco, in contrast to wild-type phaseolin (*20*). On the basis of their high degree of stability against proteolysis in vitro, BHL8 and BHL9 appeared to be the most likely of the BHL proteins to be expressed at high levels in plants. Considering amino acid composition only, BHL8 may be best suited for poultry, and BHL9 for swine.

The BHL proteins may also be helpful in improving human nutrition in areas where much of the diet comes from a staple cereal crop. The two most limiting amino acids for humans subsisting on maize are lysine and tryptophan (*21*), and these amino acids are greatly enriched in BHL8 and BHL9, relative to maize endosperm protein (Table 1).

The low expression of BHL9 in *E. coli* was surprising, but may not be relevant for predicting expression levels in plants. The stabilizing BHL9 disufide bond may not have formed in vivo in the relatively reducing environment of the *E. coli* cytoplasm, but instead probably formed spontaneously during purification. Disulfide formation would be much more likely to occur in the more oxidizing environment of the secretory pathway in a plant cell.

Versatility of the CI-2 Scaffold. BHL9 and BHL8 now provide two examples of disulfide-stabilized CI-2 derivatives that contain substitutions for over a third of their amino acids and yet retain a high degree of stability in vitro. Thus, the CI-2 platform appears to provide flexibility for solving varying nutritional problems. In proceeding from BHL8 to BHL9, one or more of the amino acid differences resulted in greater thermodynamic stability and also greater proteolytic stability against SIF, trypsin, and chymotrypsin. Although we do not know which of the numerous substitutions contributed to the increased stability in BHL9, it is thought that amino acids with branched side chains, such as threonine and isoleucine, contribute to the thermodynamic stability of proteins by decreasing the entropy of the unfolded form (*22*). The high content of these residues in BHL9 may therefore have contributed to the higher thermodynamic stability, which has been shown with other proteins to be correlated with proteolytic stability (*23*, *24*), as evident here with the SIF data of Table 2.

In conclusion, this study demonstrated that it is possible to achieve two seemingly contradictory goals in proteins engineered for nutritional enhancement—a high degree of stability against proteases in general (and therefore a potentially high expression level in plants) and yet ready digestibility of the proteins by monogastric animals. These two goals were not mutually exclusive for the BHL proteins. In the extremely low pH of the gastric fluid, the proteins were denatured with consequent exposure of proteolytic cleavage sites and rapid digestion. Alternatively, in the more neutral pH that is typical of, for example, the neutral storage protein vacuoles of a plant cell (*25*), the stable BHL proteins such as BHL8 and BHL9 were compactly folded and relatively resistant to proteases. Such compactly folded proteins would also be more likely to escape the ER-associated degradation observed with malfolded proteins (*26*).

ABBREVIATIONS USED

BHL, barley high lysine; CD, circular dichroism; CI-2, chymotrypsin inhibitor-2; GdmCl, guanidinium chloride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; UV, ultraviolet; WT, wild type.

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